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BLOOD

The Journal of The American Society of Hematology

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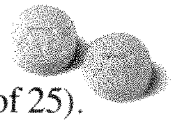

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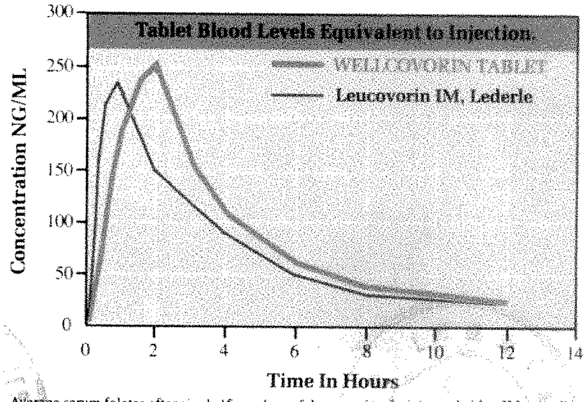
Blum MR, Guaspari A, Liao SHT, et al. Bioavailability and pharmacokinetics of oral and parenteral leucovorin. 33rd National Meeting Pharm Sci. San Diego, California, 1982, 12(2):175.



Average serum folates after single 15 mg dose of leucovorin administered either IM or orally. Adapted from Blum, et al

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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer, 41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al:* High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med, 68*:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro. Br J Haematol, 40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al:* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis, 135*(3): 493-496, 1977.

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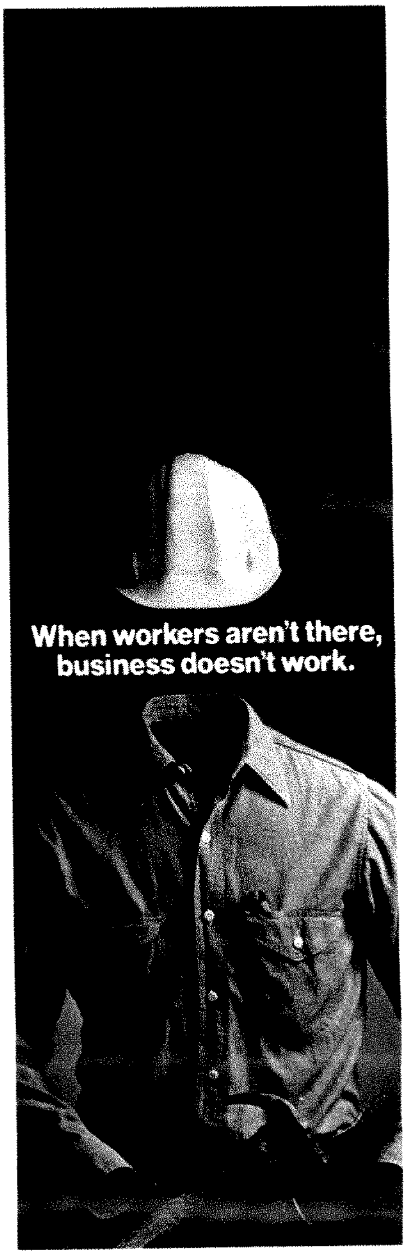

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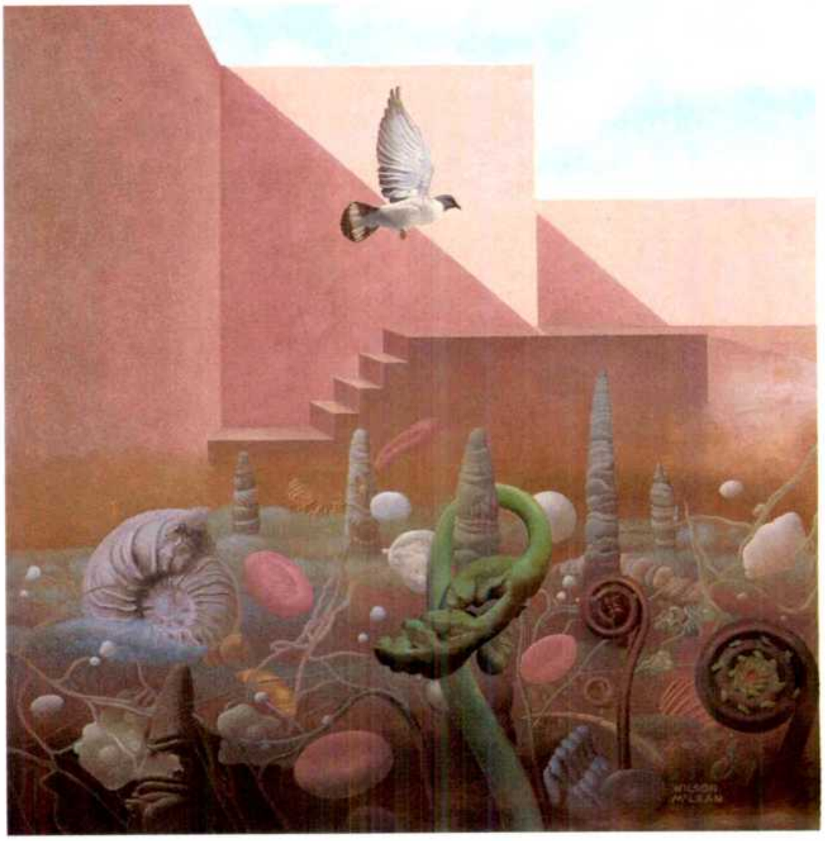

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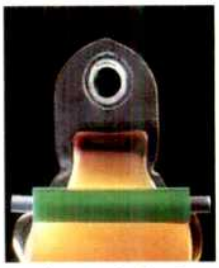

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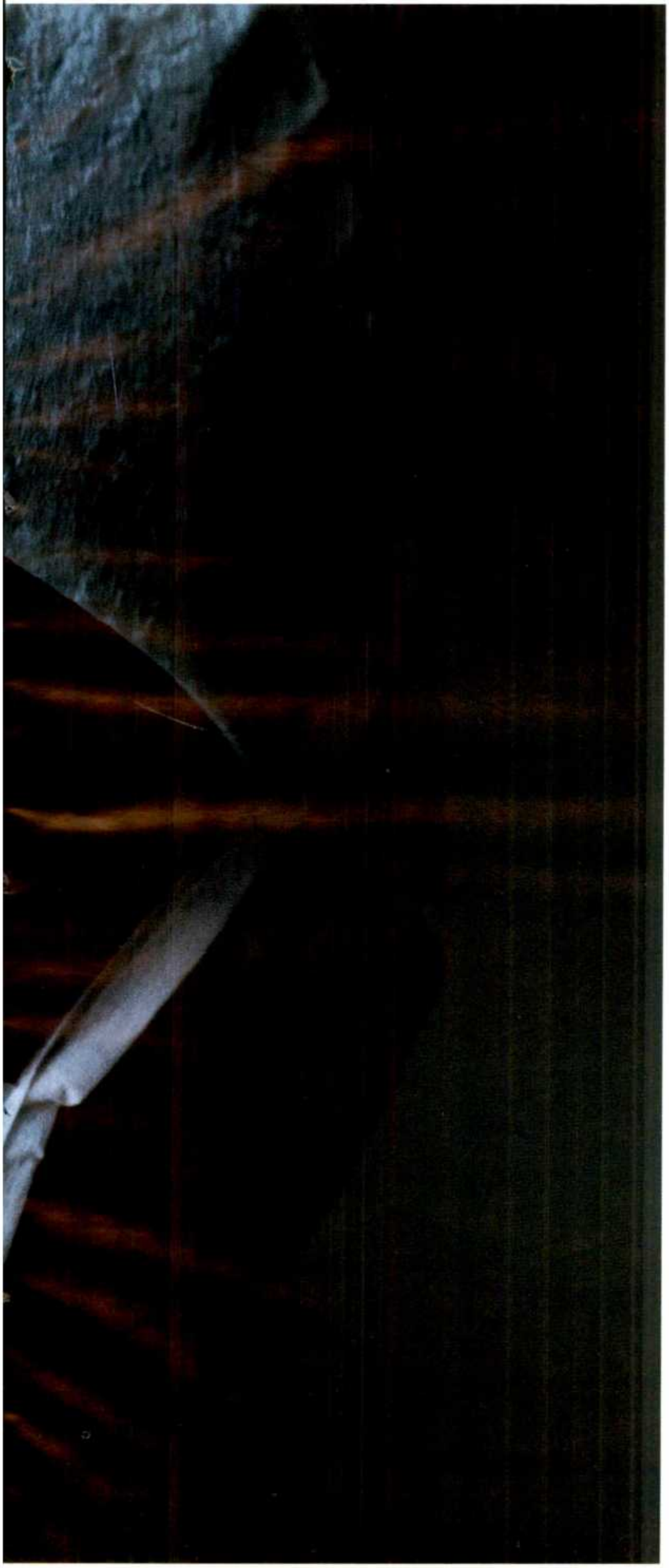

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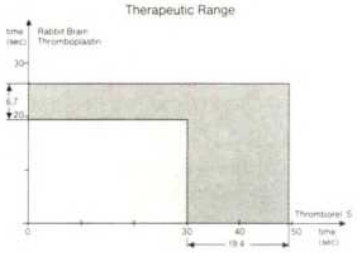

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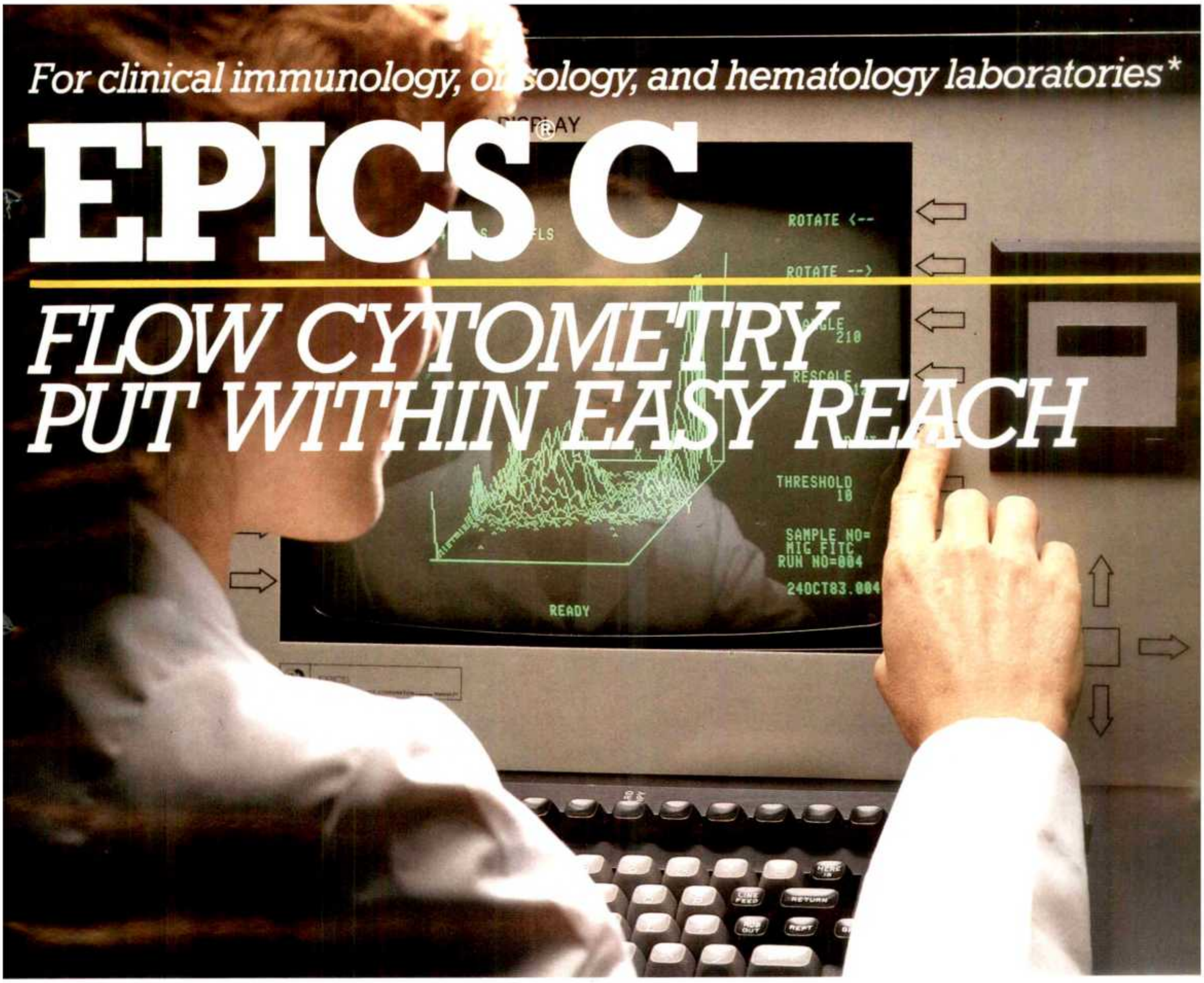

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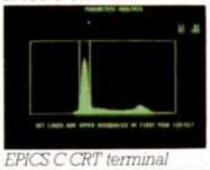

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HERPES SIMPLEX INFECTION IN THE COMPROMISED HOST:

OFTEN SERIOUS, TOO OFTEN UNSUSPECTED

Number one in a series of typical case presentations.

Esophagitis in a leukemia patient

In patients with hematologic malignancies, the visceral organ most frequently involved by herpes simplex virus (HSV) infection is the esophagus.[1] Extensive ulcerative lesions may develop on the upper two thirds of the esophagus with confluent lesions of the distal third.[2] In some cases, however, the mucosa may be grossly unremarkable even when microscopic examination shows evidence of herpetic ulcerative esophagitis.[1] Similarly, some patients may display symptoms of dysphagia and burning retrosternal pain,[3] whereas others may appear asymptomatic.[1] The diagnostic method of choice for patients with suspected herpetic esophagitis is esophagoscopy with visualization of the mucosa and procurement of cytologic and biopsy specimens from ulcerated areas.[1]

References: 1. Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **2.** Wong KK, Hirsch MS: Herpes virus infections in patients with neoplastic disease. Diagnosis and therapy. *Am J Med* 1984; 76:464-478. **3.** Lightdale CJ, Wolf DJ, Marcucci RA, et al: Herpetic esophagitis in patients with cancer: Ante mortem diagnosis by brush cytology. *Cancer* 1977;39:223-226.

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Herpetic infections may result in extensive disease and death

Immunocompromised patients are highly vulnerable to HSV infection. Such infections may interrupt therapy and even lead to death if not detected early and managed aggressively.[1] Although the initial appearance may be mild, HSV infection may progress to severe localized mucocutaneous ulcerations. The greatest threat that herpes simplex poses is an infection that disseminates from a mucocutaneous or unrecognized primary lesion and involves the lungs, liver, esophagus, or central nervous system.[1] Furthermore, herpetic ulcers frequently provide an entry for secondary bacterial and fungal pathogens.[2]

Infections may not be diagnosed correctly without virologic study

Some investigators claim that the actual incidence of serious HSV infection is probably higher than reported because "the possibility of herpetic disease is insufficiently considered and appropriate diagnostic viral studies are often not carried out."[3] One study, for example, found histocytologic evidence of visceral herpes infection in 56 patients even though herpetic involvement was not suspected in any of the patients prior to autopsy.[2]

ZOVIRAX I.V. halts viral replication and speeds healing even in debilitated patients

ZOVIRAX I.V. has been shown to be a highly effective treatment for severe mucocutaneous HSV infection in immunocompromised patients. In controlled trials, ZOVIRAX I.V. rapidly halted viral shedding — within 3 days in most patients — and significantly shortened healing time as well as duration of pain.[4,5] Furthermore, ZOVIRAX I.V. may allow patients to return home sooner and enable patients with intraoral lesions to resume normal eating habits.[6]

ZOVIRAX I.V. is well tolerated by immunocompromised patients

A multicenter collaborative trial of ZOVIRAX I.V. in immunocompromised patients reported that: "Clinical benefits were obtained without appreciable toxicity. The most common reactions were irritation of peripheral veins used for drug infusion and a low incidence of rash.... Significant renal or hematologic toxicity did not occur, even in these seriously ill patients who were commonly receiving other drugs concomitantly."[5]

Note: Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes. Please see brief summary for more information.

References: **1.** Feldman S, Cox F: Viral infections and hematological malignancies. *Clin Haematol* 1976;5:311-328. **2.** Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **3.** Muller SA, Herrmann EC, Winkelmann RK: Herpes simplex infections in hematologic malignancies. *Am J Med* 1972;52:102-114. **4.** Wade JC, Newton B, McLaren C, et al: Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation. *Ann Intern Med* 1982;96:265-269. **5.** Meyers JD, Wade JC, Mitchell CD, et al: Multicenter collaborative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. *Am J Med* 1982;73(1A):229-235. **6.** Data on file, Burroughs Wellcome Co.

An effective response to a life-threatening infection

ZOVIRAX® I.V. INFUSION

(acyclovir sodium) sterile powder

An effective response to a life-threatening infection

ZOVIRAX® I.V. INFUSION

(acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously, or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: 1. C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** J.C. Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am. J. Med. 73(1A): 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. *33*:1452-1463, 1973.

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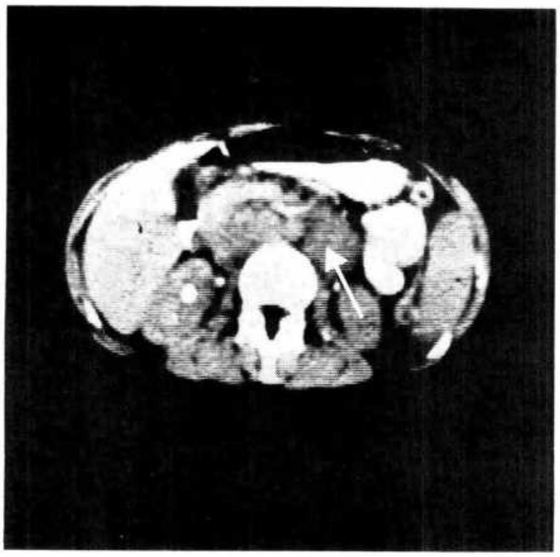
(sterile bleomycin sulfate)
Proven Effective in Non-Hodgkin's Lymphoma

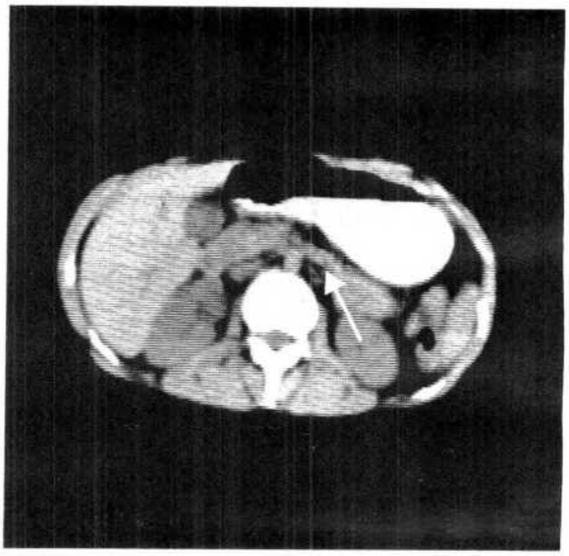
The newer Blenoxane containing combinations resulted in high complete response rates and extended disease-free survival.[1-7]

Abdominal/Pelvic CT
February 25



Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al:* Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al:* Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen SL, *et al:* Combination chemotherapy of advanced diffuse histiocytic lymphoma with the six-drug COP-BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

*Case information on file at Bristol-Myers Oncology Division

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Blenoxane® ...*Integral Component of Chemotherapy Regimens in Non-Hodgkin's Lymphoma*

(sterile bleomycin sulfate)

Since Blenoxane is virtually non-myelosuppressive, continuing therapy with certain Blenoxane containing chemotherapy regimens (e.g. BACOP) can prevent tumor regrowth between cycles of other chemotherapy regimens while allowing for bone marrow recovery.[*2,3,4,6]

Blenoxane can produce, with cumulative doses, a syndrome of pulmonary fibrosis. It is usually dose-related, being seen infrequently in patients whose total dose of Blenoxane is less than 400 units.

Efficacy...Yesterday, Today, Tomorrow

Blenoxane®
(sterile bleomycin sulfate)

M-BACOD

4. Skarin AT, Canellos GP, Rosenthal DS, *et al:* Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
5. Anderson KC, Skarin AT, Rosenthal DS, *et al:* Combination chemotherapy for advanced non-Hodgkin's lymphomas other than diffuse histiocytic or undifferentiated histologies. *Cancer Treat Rep* 68:1343-1350, 1984.

m-BACOD

6. Skarin A, Canellos G, Rosenthal D, *et al:* Moderate dose methotrexate (m) combined with bleomycin (B), Adriamycin (A), cyclophosphamide (C), Oncovin (O) and dexamethasone (D), *m*-BACOD, in advanced diffuse histiocytic lymphoma (DHL). *Proc Am Soc Clin Oncol* 2:220, 1983.

MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

Blenoxane® *(sterile bleomycin sulfate)*

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

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Edited by

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REVIEW

Lineage Promiscuity in Hemopoietic Differentiation and Leukemia

By M.F. Greaves, L.C. Chan, A.J.W. Furley, S.M. Watt, and H.V. Molgaard

An increasing number of reports document instances in which individual leukemic cells coexpress markers normally believed to be restricted to a single lineage. This has been interpreted by McCulloch and colleagues as aberrant programming or lineage infidelity and contrasts with earlier suggestions that lineage fidelity of gene expression was usually maintained in leukemia. We argue that several examples of infidelity are suspect on technical grounds, whereas others are bona fide and require explanation, eg, partial rearrangements and expression of Ig heavy-chain and/or T cell receptor genes in inappropriate cells and terminal deoxynucleotidyl transferase in leukemic myeloblasts. Individual examples of truly aberrant gene expression may well occur in leukemia but with insufficient regularity to be of general significance. We suggest that verifiable and consistent examples of apparent lineage infidelity do not reflect genetic misprogramming but rather the existence of a transient phase of limited promiscuity of gene expression occurring in normal bipotential or multipotential progenitors and able to be preserved as a relic in leukemic blast cell populations that are in maturation arrest. This alternative explanation has interesting implications for mechanisms of hematopoietic differentiation and leads to some testable predictions.

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IMMUNOLOGIC, enzymatic, and molecular investigations of human leukemias and lymphomas support the notion of a unicellular (monoclonal) origin and an apparent maturation arrest or uncoupling of proliferation and differentiation.[1-3] From a consideration of the composite phenotypes of leukemia cells in comparison with normal populations it has been possible, in the majority of cases, to designate the predominant lineage and cell type involved and at least to speculate as to the possible target cell for clonal expansion.[4-6] Such analyses form the basis of new classification schemes and diagnostic programs and have prognostic relevance in the context of particular therapeutic regimes.[7-10]

Leukemic phenotypes are not perfect replicas of normal ones. In addition to specific chromosomal changes and alteration in the structure and/or control of particular genes, leukemic cells may show some asynchrony of phenotypic expression in comparison to their equivalent maturation compartment in normal tissue. In a study of the composite immunophenotype in 82 cases of T cell leukemia, 26 showed some asynchrony or abnormal combination of T cell markers with respect to intralineage maturation status.[11] Additionally, all cases of thymic or T acute lymphoblastic leukemia tested expressed high-density HLA (A, B) combined with nuclear terminal deoxynucleotidyl transferase (TdT) in individual cells,[11,12] whereas in the normal thymus, TdT-positive cells have very low density HLA (A, B), the density of this glycoprotein increasing with further T cell maturation and loss of TdT activity.[13] Similarly, in leukemia of B cell precursors, ie, common acute lymphoblastic leukemia, a substantial proportion of cases are cytoplasmic μ chain positive/TdT-positive,[14,15] whereas in normal populations, this is an extremely rare (though detectable) cellular phenotype,[16] pre–B cells presumably losing TdT prior to detectable μ chain synthesis. Significantly, these distortions in phenotype were much more pronounced in cell lines established from acute lymphocytic leukemia (ALL) patients (usually in relapse) compared with uncultured cells studied at diagnosis.[11]

The minimal deviation or asynchrony of maturation-linked phenotype usually observed in leukemic cells at diagnosis is not surprising if the arrest is not an absolute bar to maturation but, as proposed, a regulatory uncoupling of variable stringency.[17] Lineage specificity, on the other hand, has appeared to be rather faithfully conserved in leukemia and lymphoma. For example, the gp100/common acute lymphoblastic leukemia antigen (CALLA, CD-10) structure[17,18] is non–lineage specific[19] but is selectively expressed on certain cell types in concert with maturation status. Although granulocytes may express CALLA weakly,[20] myeloid progenitors are unreactive[21] in contrast to putative

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lymphoid precursors in bone marrow and thymus that are CALLA-positive.[16,22,23] In a survey of over 2,000 cases of leukemia with the anti-CALLA monoclonal J-5,[23] 802 out of 1,072 (74.8%) ALL were reactive compared with only 14 out of 573 (2.4%) cases of acute myelogenous leukemia (AML). More significantly on review of the latter 14 cases, three cases had dual lymphoid-myeloid populations, ten had no unequivocal evidence for myeloid cytochemistry, and one was originally an AML but relapsed as ALL. Nine of these pseudomyeloid leukemias were treated with therapy for ALL, and eight responded well.[23] Clearly, therefore, it is exceedingly rare for leukemic myeloblasts to express illegitimately the CALLA marker. Glycophorin is an erythroid cell–specific membrane glycoprotein expressed on most but not all erythroleukemias.[24] When a monoclonal antibody, LICR.LON.R10,[25] was used to screen 329 cases of ALL, only two had positive blasts.[24] Review of these cases revealed that although both had a lymphoblast morphology, they did not express an ALL immunophenotype and were almost certainly cryptic erythroleukemias.

On the basis of these considerations it was suggested that lineage fidelity was the norm in leukemia and that the stabilization of a normally transient progenitor or precursor cell phenotype was a critical event in leukemogenesis.[3,26,27]

LINEAGE INFIDELITY IN LEUKEMIA: IDENTITY CRISIS FOR CELLS, MONOCLONAL ANTIBODIES, OR INVESTIGATORS?

The idea that leukemic cells are phenotypically similar to normal hematopoietic progenitors or subsets has now been challenged with an increasing number of reports, in particular from McCulloch and colleagues, claiming to have demonstrated lineage infidelity of gene expression (Table 1). Infidelity is defined here according to McCulloch's arguments[59] as a misprogramming of differentiation in leukemia with the consequence that there is coexpression on individual cells of markers normally found only on cells belonging to different but related lineages, eg, lymphoid plus myeloid or granulocytic plus erythroid. McCulloch refers to the former as interlineage infidelity and the latter as intralineage (myeloid) infidelity.[59] Since the term intralineage infidelity might be confused with asynchronous maturation in leukemia (see above), we do not use it here and adhere to the simple definition of interlineage infidelity given previously. This situation should be distinguished from cases of leukemia (often termed biclonal) in which cells of two lineages (eg, myeloid and lymphoid) coexist simultaneously or appear sequentially in an individual patient and presumably reflect the transformation of a common precursor whose progeny have undergone further differentiation.

Table 1. Summary of Reported Examples of Apparent Lineage Infidelity in Leukemia

1	20 acute leukemias studied	
	A 6 cases: factor 8 or spectrin + My-1 or OKM1 binding	
	B Ig + My-1 binding	28
2.	MOLT-3, T-ALL cell line + 5-azacytidine → binding of My-1 and 80H.5 (CDw 15, anti-X hapten) and other antibodies assumed to be unreactive with T cell leukemia—J-5 (and CALLA) and BA-2 (anti-p24).*	29
3.	K562 cell line spectrin + My-1 binding	30
4	Monocytoid cytochemistry in TdT$^+$ lymphoblasts carrying the (4,11) translocation	31
5.	Reactivity with IG10 (anti-X hapten) plus inducible monocytoid features in acute leukemic cell line with the (4,11) translocation and the composite phenotype of a B cell precursor ALL.	32
6	T cell antigen gp65-67kd/T1 (CD-5) expressed in B chronic lymphocytic leukemia (B-CLL).	33
7.	Coexpression of the E rosette receptor, gp50/T11 (CD-2) with monoclonal Ig in lymphoid leukemia.	34–36
8.	Coexpression of T11 with the myeloid marker MY9.	37
9.	Expression of T11 structure (gp50) on leukemic myeloblasts (4/23 AML patients)	38
10	Expression of the granulocytic antigen anti-X hapten antibodies (VIM-D5, LeuM1) on ALL cells	39
11	Coexpression of several T cell antigens (T3, T4, T6, T8, T11) with membrane Ig.	40, 41
12	Expression of myeloid enzymes or myeloid antigen (CDw 13, antibody MCS-2) on ALL cells (three cases) †	42
13.	Expression of T cell antigen gp40/CD-7 (antibodies 3A1, WT1) on leukemic myeloblasts	44–46
14.	Coexpression of T cell and myeloid markers CD-1/VIM-D5 (anti-X hapten), CD-3/VIM-D5, TdT/VIM-D5.	47
15.	Presence of nuclear TdT in leukemic myeloblasts	47–51
16.	Ig gene rearrangement in T leukemic cells or cell lines.	52–54
17	Ig gene rearrangement in myeloid leukemia or myeloid cell line (ML-1).	55–57
18	T cell receptor rearrangement in non-T leukemic cells.	58

*An assumption here that gp100/CALLA and p24/BA-2 are not expressed by T cells and thymic leukemias is clearly false (see refs 11, 23)

†In two of these three cases the presence of myeloid markers in lymphoblasts was not clearly demonstrated.[43]

We regard this issue as being of considerable interest since if infidelity were relatively common it would largely nullify the views summarized previously and might carry important implications for both leukemogenesis and normal differentiation as well as for clinical applications of phenotypic markers. In this article we analyze the evidence presented in support of this concept and propose an alternative interpretation of the data.

It must first be acknowledged that, if leukemic cells very occasionally express an unexpected or ectopic product that is discordant with the overall, composite phenotype, then this should cause no great surprise, especially if it occurs in concert with malignant progression and multiple chromosomal alterations or following prolonged culture in vitro. On the other hand, it is rather unlikely that such an infrequent abnormality can shed any light on fundamental and common mechanisms of leukemogenesis and differentiation. Consistent phenotypic features, at least within a subtype of leukemia, are therefore of more interest. Although some of the reported claims for lineage infidelity in leukemia are anecdotal and have not been substantiated, others demand more attention. Our view is that many of the reported examples of illegitimate gene expression are suspect since they rely upon presumptions of lineage specificity of the marker used that are either unsubstantiated or clearly false. This is most evident with certain monoclonal antibodies in the CDw 15 set[61] that have been used to detect inappropriate expression of granulocyte-associated antigens[28,29,32,38,40,47] (Table 1). Although the original reports of these reagents clearly demonstrated the preferential reactivity of relatively mature myeloid cells with antibodies such as My-1,[62] VEP8/9,[63] MI/N1,[64] VIM-D5,[65] 80.H5,[66] HL-C5,[67] and IG10[68] (Fig 1), it is known that these all detect the same carbohydrate antigen (3-fucosyl-N-acetyllactosamine or X hapten)[69,70] that far from being lineage specific is cross-reactive or identical to the SSEA-1 antigen of teratocarcinomas and certain embryonic cells and expressed in a wide variety of tissues (normal and malignant) and secretions[70] (Fig 1). Within the human hematopoietic system, the X hapten determinant has been identified using some of the aforementioned antibodies on multipotential progenitors and committed progenitors of several lineages[71] and is present in a cryptic but easily exposable form on acute lymphoblastic leukemia cells.[72] Clearly, the presence or exposure of such an antigen on nongranulocytic cells cannot be regarded as evidence for lineage infidelity of gene expression.

Other markers used in reports of infidelity (Table 1) are also suspect. Polyclonal antibodies to spectrin are very unlikely to be erythroid cell specific as assumed[28,30] since spectrin is known to be expressed in a wide variety of cell types including lymphocytes.[73] Neither are several of the T cell differentiation markers restricted to cells of the T lineage as also assumed. This is especially true of Leu-1/OKT1 (CD-5), which was unexpectedly found on B-CLL cells[33] but later identified on an infrequent subset of normal B cells.[74] The situation with other T cell–associated determinants is less clear although T4 (CD-4) is expressed by macrophages[75] and T6 (CD-1) by Langerhans cells in the skin.[76] Several authors report that the T11 (CD-2), E rosette marker of T cells can be expressed by monoclonal (κ or λ) B cell leukemias or leukemic myeloblasts (see Table 1). Expression of the T11 marker on normal, activated B cells has been reported,[36] but its presence on normal myeloid cells has yet to be demonstrated.

Other observations that might reflect infidelity cannot easily be refuted on the basis of reagent specificity or exclusive use of cell lines. These include (see Table 1) the presence of nuclear TdT in leukemic myeloblasts, the presence of gp40/CD-7 T cell–associated proteins on leukemic myeloblasts and, perhaps most strikingly, the rearrangement and/or expression of an immunoglobulin or T cell receptor *β* gene in inappropriate lymphoid or myeloid leukemic cells. We regard these observations as particularly instructive and will discuss them in more detail.

Several other combinations of markers reported to be expressed on individual cells appear to be abnormal. These are, however, mostly on selected, long-established cell lines, in some cases treated with phorbol ester (see Table 1), and the general significance of such observations for gene expression in leukemia is difficult to ascertain.

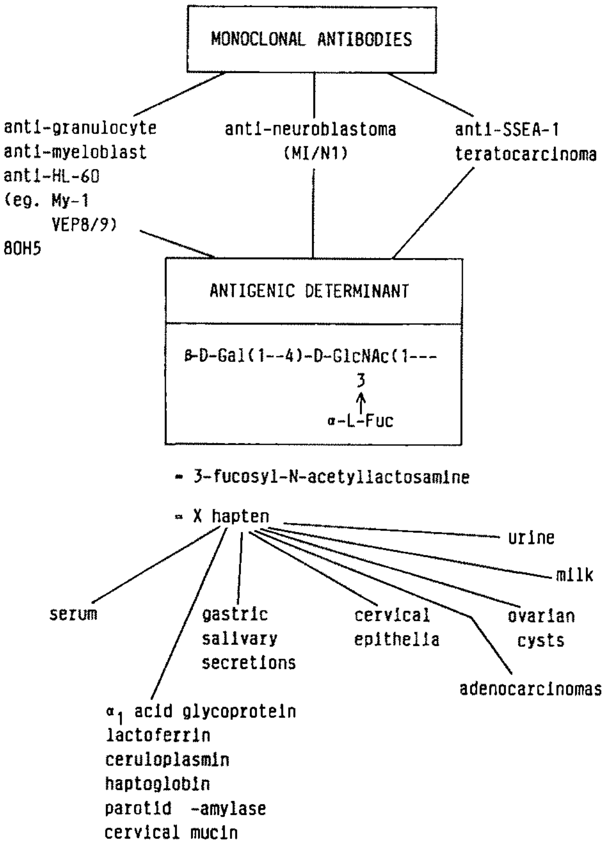

Fig 1. Widespread expression of the X hapten antigen, 3-fucosyl-N-acetyllactosamine.

Finally, McCulloch argues that the credibility of the existence of misprogrammed phenotypes is reinforced by the finding that AML patients with such aberrant cells have an inferior prognosis.[59] Even if we accept that the clinical observation, based upon rather few patients (a third of whom were unresponsive and in relapse at the time of study), is correct, the argument is itself unacceptable. A particular phenotypic constellation of markers may well provide a prognostic correlate and, in one sense, is therefore real rather than imaginary; whether that set of markers is normal or bizarre in the context of normal hematopoiesis is an entirely separate issue.

The difficulties pervading these studies, and many others concerned with leukemic cell phenotypes, revolve around presumptions made both about the specificity of markers or reagents used and of the anticipated phenotypes of normal equivalent cells. Three technical problems seem to stand out:

1. An insufficient recognition of the limited screening and often misleading nomenclature that has been applied to many antibodies prior to their widespread availability is the first technical problem. With more stringent and extensive specificity evaluation, it becomes evident that remarkably few antigenic determinants appear to exist that are entirely

restricted to a given cell type or lineage. The exclusive characteristics of cells may lie more in their composite mosaic of gene expression within which a small number of specialized function-related molecules may represent unique identity tags.

Clear examples of this type of problem are provided by studies with the T cell–associated markers identified by the monoclonal antibodies OKT9 and OKT10 (and other equivalent antibodies) These were raised against thymocytes (and T-ALL cells) and reported to identify thymocyte-associated molecules and discrete stages of T cell differentiation.[77,78] Although the original data are not refuted, it was another two years before it was widely appreciated that OKT9 and OKT10 were not T cell–specific structures but were glycoproteins with widespread expression associated with cell activation. T10 is expressed on hematopoietic cell progenitors and activated mature lymphocytes and in plasma cells,[79,80] and the T9 structure is the transferrin receptor associated ubiquitously with proliferation in vertebrate cells.[81] Similarly, the IL2 receptor identified by the anti–T cell activation antigen (anti-TAC)[82] was widely anticipated to be T cell lineage specific but is now clearly demonstrated to be expressed on some activated normal B cells and B leukemia cells.[83,84]

2. A common presumption among users of monoclonal antibodies is that these reagents are monospecific and will react with only a single determinant. This view contradicts one of the fundamental tenets of immunology. Antigenic specificity is never absolute and is a matter of relative affinity of antibody-combining sites. Antibodies, whether derived from cloned hybridomas or not, will be potentially able to cross-react with a wide variety of related and unrelated structures and, in addition, may bind via their Fc region rather than through the antibody-combining sites. The only safeguards against these problems are to know something about the chemical characteristics of the primary antigenic epitope recognized by the antibody and the structure(s) with which it is associated both in the normal and unexpected circumstance and also to use immunoglobulin class-specific controls for binding studies. Several of the aforementioned studies (Table 1) lack appropriate controls, and in only few cases has biochemical similarity or identity to the primary antigen been established.[38,44]

3. Insufficient knowledge of the immunophenotypes of normal lymphocyte subsets and the various stem cell/progenitor cell populations to which leukemias or lymphomas may correspond severely restricts the interpretation of leukemic cell phenotypes. Although this represents a considerable handicap, the identification of apparently novel markers on subsets of leukemias and lymphomas has, paradoxically perhaps, been successfully exploited to identify relatively infrequent normal cells and molecules associated with them that would otherwise have been inaccessible. Pertinent examples of the latter include identification of cells of the common ALL phenotype in normal bone marrow and fetal liver,[15,23] the B-CLL equivalent in lymph node,[74] the Sternberg-Reed (Hodgkin's) cell equivalent (reactive with antibody Ki-1) in normal lymph nodes,[85] the Burkitt's lymphoma antigen in normal lymph node germinal centers,[86] and the discovery of a stem cell/multipotential progenitor cell–associated glycoprotein based upon selective expression of an antigenic determinant (detected by the antibodies MY10, 3C5) shared by immature lymphoid and myeloid leukemias.[87–89]

We conclude that the evidence presented so far for misprogramming of differentiation or lineage infidelity of gene expression as a consistent feature of leukemia is in part invalid and unconvincing overall. We do not consider the issue clear-cut or resolved, however, since some examples of apparent infidelity are difficult to refute on the grounds of reagent specificities (see the preceding material), and the counterargument for fidelity, though compelling, could be biased by emphasis on lymphoid malignancies or selective use of markers that are subject to very stringent control following lineage commitment.

A more particular reason for expressing this reservation, however, concerns considerations of acute leukemias that might arise in cells that are not themselves committed to a single lineage and whose progeny also become arrested at a very early stage of hematopoiesis. Since we are still ignorant of the mechanisms by which bipotential or multipotential cells adopt lineage options available to them, it is premature to presume that all genes whose activity is normally associated with particular individual lineages will be expressed exclusively and in an all-or-nothing fashion.

PROMISCUITY NOT INFIDELITY OF GENE EXPRESSION IN ACUTE LEUKEMIA AND IN NORMAL HEMATOPOIESIS?

Much of the argument surrounding the varied interpretations of leukemic cell phenotypes involves the presumption that all normal hematopoietic and lymphoid cells show strict lineage specificity. This view unfortunately embodies a rather circular argument since the discovery that a marker is not, after all, lineage specific may disqualify it as an individual lineage indicator. Additionally, monoclonal antibodies raised against leukemic cells and found on initial screening to bind to both lymphoid and myeloid cells have frequently been considered of only marginal interest. As McCulloch points out,[59] there is sound reason to believe that lineage fidelity is maintained for postcommitment events, but considerable uncertainty pertains to earlier events. It is possible that some markers that are exclusively expressed in a lineage-restricted fashion on precursor cells committed to a single lineage are also transiently coexpressed on some or all multipotential cells and perhaps briefly on the progeny of these cells following the initiation of commitment but prior to full or irreversible determination. Could not, therefore, some limited degree of lineage promiscuity precede lineage fidelity as a normal course of events? If this were correct and a leukemia arose in such a precommitment phase of hematopoiesis and was subject to a relatively stringent maturation arrest, then appropriate marker analysis might reveal mixed lineage phenotypes on individual cells (Fig 2). Fidelity (ie, faithfulness to the original) would be preserved in such leukemias as lineage promiscuity but easily misread as lineage infidelity.

A similar argument with respect to normal hematopoiesis

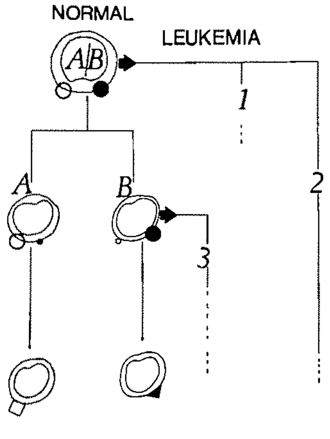

Fig 2. Restricted promiscuity of lineage-associated gene expression in hematopoiesis and leukemia. Key: A/B, bipotential progenitor cells; A B, unilineage-restricted progenitor cells; ● O, early lineage markers functionally involved in the commitment process; • o, residual expression of early markers in inappropriate lineage (relics); □ ▲, late markers involved in mature cell function; ➧ target cell for leukemogenesis; and ———-, maturation of leukemia clone (in vivo). A bipotential progenitor is shown coexpressing early (● O) but not late (□ ▲) markers of lineages A and B. The early markers may be functionally involved in the commitment process (see text). Not all multipotential (A/B) cells need express both markers simultaneously, especially if the activation process is stochastic. Immature cells committed to lineage A or B may still express, at a low level, the inappropriate gene product as a relic of parental promiscuity (small symbols, • o, on A B). The phenotypic pattern of gene expression with respect to these same markers in leukemia depends upon the level of target cell transformation (and clonal selection) and the maturation competence (in vivo) of the leukemic clone. Three types of leukemia are indicated in the Fig. In 1 and 2, the target cell is a bipotential progenitor (A/B). In leukemia 1, differentiation arrest is stringent; in leukemia 2, maturation is relatively normal (compare chronic granulocytic leukemia) [CGL]). B lineage-restricted cells are indicated as the target for leukemia 3. In leukemia, lineage promiscuity of the phenotype is most likely to be found in leukemia type 1.

was proposed by Till[90] who suggested that many lineage-associated markers might be coexpressed at a low level on multipotential progenitors and then selectively lost as cells differentiate and mature. Berridge and Okech subsequently produced some experimental support for this concept using absorbed heteroantisera to murine hematopoietic cells.[91] Although it is conceivable that all potentially available genes are expressed at a low level in progenitor cells, in its extreme form this hypothesis is unlikely to be correct since several lineage- and maturation-associated markers are undetectable on multipotential progenitors (eg, CALLA, glycophorin, T3). Nevertheless, limited or selective promiscuity could occur with respect to genes that are among the first to be expressed in a lineage and whose activity is perhaps functionally involved in lineage commitment (Fig 2).

IMMUNOGLOBULIN AND T CELL RECEPTOR GENE REARRANGEMENT AND EXPRESSION IN ACUTE LEUKEMIAS

In considering the possibility of limited promiscuity of gene expression in early hematopoiesis, we have in mind particularly the finding of immunoglobulin gene rearrangement and/or expression in T cell leukemias[52–54] and myeloid leukemias[55–57] and T cell receptor gene rearrangement and/or expression in some non–T cell leukemias.[58] These genes code for protein molecules with a known function that is cell type–unique in the same sense that hemoglobin is a specialized product of red cells. It is therefore rather striking to find that cells expressing one of these molecular markers may have a composite phenotype indicating that it belongs to a lineage not normally associated with rearrangement or expression of the gene in question.

A clue to the possible significance of these unexpected observations comes from a more detailed scrutiny of their molecular nature. Earlier studies in the mouse demonstrated that normal mouse thymocytes as well as some cytotoxic T cell clones, leukemic T cells, and myeloid cells have immunoglobulin heavy-chain gene rearrangements.[92–96] Transcripts of various sizes were also reported, and in one case these were shown to result from DJ joining in the absence of V regions.[95] Parallel studies on Abelson virus–transformed B cell precursors in fetal liver by Alt and colleagues[97,98] also revealed that DJ joining, with a corresponding truncated messenger RNA (mRNA) and protein, probably precedes recombination involving V region genes in normal B cell ontogeny. Yancopoulos and Alt also report that transcription of unrearranged V_H gene segments is prominent in cells undergoing V_H-to-DJ_H rearrangement and, in contrast to DJ_H rearrangement, is B cell specific.[99] In our studies on thymic ALL, three out of 13 cell lines and fresh samples investigated had a single μ gene allele rearranged.[53] Subsequent analysis of these rearrangements were suggestive of DJ joining (Molgaard and Ford, unpublished observations, 1985). Significantly, no non–B cell leukemias have been reported with rearrangement of κ or λ light-chain genes—events that occur later in pre–B cell ontogeny than rearrangement of both heavy-chain alleles.[52]

A very similar story is now emerging with respect to the T cell receptor β chain gene.[100] The latter has an immunoglobulin-like VDJC region suprastructure[101,102] and undergoes clonal rearrangement that can be visualized on leukemic cells by Southern blot analysis of DNA.[103] Data from both mice[104–107] and humans[106] indicate that immature thymocytes, both normal and leukemic, transcribe a 1.0kb mRNA, which is probably the product of a DJC β gene lacking a V region. Contrary to the results of Royer et al,[107] we find that the most immature T-ALL we can identify (with the phenotype: T11 + or −, T6−, T3−) have β gene rearrangement and 1.0kb transcripts.[108] Significantly, however, some of these leukemias also have a μ gene rearrangement.[108] Moreover, we also detect β gene rearrangement and/or a 1.0kb transcript of the β gene in a few leukemias that are immature myeloid or pre-B in composite phenotype.[58]

One explanation offered for these observations is that in T and B cell precursors there may be "cross talk" of the recombinases or other enzymes involved in DJ joining of immunoglobulin and the T cell receptor β gene.[109] Although this seems a plausible hypothesis, it raises the intriguing question of what makes a T cell a true T cell and a B cell a true B cell. One possibility is that DJ joining can occur in uncommitted cells and function as a necessary but insufficient genetic step in lymphoid lineage commitment. Subsequent successful recombination with a V region may then produce a protein product that irreversibly commits a cell to the B or T pathway in part by switching off further rearrangements of genes belonging to the alternative pathway and in a similar manner repress rearrangements of its own alternative allele to ensure allelic restriction of the product.[99,110,111] Unsuccessful recombination with the VIg or V β gene might permit alternative lineage possibilities to be adopted (ie, including myeloid). Since most myeloid and B lineage leukemic cells do not show evidence for DJ β rearrangement (which should be irreversible and hence detectable), we conclude, however, that lineage promiscuity with respect to this marker is not a necessary or even very common event in multipotential stem cells.

OTHER EARLY MARKERS OF HEMATOPOIETIC DIFFERENTIATION

The activity of other lineage-associated genes might similarly be required as part of the initiation process of commitment and coexpressed in some multipotential cells. We might expect this to be less likely for lineage-associated markers that arise during maturation than for molecules that arise and are expressed early in a lineage. Two early markers of human hematopoietic cells that are interesting in this respect are TdT and the T lineage-associated antigen gp40/CD-7 detected by the monoclonal antibodies 3A1,[46] WT1,[45] and Leu-9.[112]

TdT is expressed in leukemias and normal cells of the pre-B and pre-T (thymic) phenotype, is absent in mature lymphocytes,[113,114] and is postulated to play a role in increasing the diversity of immunoglobulin and T cell receptor genes by the random addition of nucleotides in the N region.[115,116] Although TdT has proved a very useful marker for acute lymphoblastic leukemia and lymphoid blast crisis of CGL, its expression is not limited to ALL. Several reports of TdT-positive AML have been published (Table 1), and in two extensive surveys of acute leukemias, 5% to 10% of cases of AML were found to be TdT-positive.[48,49] In several individual cases it has been unambiguously demonstrated that TdT and a myeloid marker (eg, myeloperoxidase or myeloid antigen) coexist in the same cells.[47,50,51] We have been reluctant to refer to this as lineage infidelity, however, since we have had no way of assessing the TdT status of appropriate control cells. It is conceivable that TdT could be induced very early in hematopoiesis in preparation for the modification of immunoglobulin and T gene rearrangements but be carried over and transiently expressed in some cells that adopt myeloid commitment. The latter might, especially if arrested in their maturation, continue to express TdT as a relic of their prior promiscuity. In contrast to TdT-positive AML, no TdT-positive CGL have been recorded in chronic-phase granulocytes despite the origin of this leukemia in pluripotential stem cells. Blast crisis of CGL that arises in the progenitor compartments with associated maturation arrest can, however, be TdT-positive.[49,117,118] We assume, therefore, that with progressive maturation of leukemic or normal cells expression of any inappropriate genes could be switched off.

Although there is very little evidence for expression of most T or B cell maturation-linked markers (eg, T3, B4) on myeloid leukemia cells, we were struck by the observation that the T lineage-associated antigen that appears to be expressed before all other described T markers in T cell differentiation—gp40/CD-7—is also coexpressed on 5% to 10% of myeloid leukemia cells.[45,46] These were not coincident with the TdT-positive cases.[45] The binding of anti-gp40 antibody to leukemic myeloid cells was ascribed to Fc activity,[46] and although this may have been the case for those AML with a monocytic component, we were able to demonstrate the presence of the gp40 molecule on leukemic myeloblasts.[44] When normal multipotential myeloid progenitors were screened using the fluorescence-activated cell sorter,[119] none had a strong or unequivocal expression of the CD-7 antigen, but a proportion (10% to 23%) of colony-forming cells (CFU-mix, BFU-E, CFU-meg, and CFU-GM) were separated into the weakly staining fraction. These observations are difficult to interpret without any clues as to the function of the CD-7 molecule. Expression of the latter may, however, like DJ rearrangements and TdT, be a marker for lymphoid lineage (T in this case) initiation or availability expressed prior to irreversible commitment.

MODELS OF LINEAGE COMMITMENT AND DIFFERENTIATION

These issues impinge directly on fundamental questions of hematopoietic differentiation and cell type–specific control of gene transcription. Several models for self-renewal and differentiation of multipotential stem cells have been proposed.[120] The major competing models of hematopoietic differentiation predict that either (1) adoption of a lineage commitment at a genetic level is indifferent to external, directive influences and follows an intrinsic program that is either stochastic (probabilistic) or deterministic (ordered) in nature[120–124] or (2) that the choice of commitment to a particular lineage is intrinsically flexible, the outcome being determined or imposed by external, microenvironmental influence,[125] eg, growth factors[126–127] and/or stromal elements[128,129] or other positional information.[130]

If, as we propose, certain early lineage-associated markers are promiscuously coexpressed on bipotential or multipotential cells (and their corresponding leukemias), then these might themselves function to advertise the accessibility of particular lineage options. Such an arrangement could provide a compromise between the two conflicting theories of commitment by incorporating features of both in sequence. Activation of particular early genes could arise intrinsically, in a programmed or randomly generated fashion, and indicate spontaneous initiation of the commitment process in one or more lineages. The particular lineage option endorsed in individual cells might subsequently be determined both by the particular pattern of markers expressed (and hence lineages available) and by the balance of external influences. We are not aware of any data that would refute this suggestion.

In respect to this model, prime candidates for promiscuous expression in addition to DJ joining in Ig heavy-chain genes and the T cell receptor genes as previously discussed would be cell surface receptors for factors that regulate growth and maturation early in a lineage, eg, granulocyte/macrophage (GM)-CSF and as yet undiscovered regulators of early lymphoid development. Both Till[90] and van Zant and Goldwasser[126] have previously suggested that uncommitted progenitors might be expected to express concomitantly or sequentially receptors for growth factors that regulate growth and maturation in a lineage-restricted fashion. Some functional studies by Metcalf and Burgess involving single-cell manipulations[127] and other studies on enriched progenitor populations[131] suggest that this could well be the case for GM colony-forming cells. Since several hematopoietic growth factors have recently been molecularly cloned,[132,133] it should soon be possible to test in vitro whether their receptors are coexpressed on bipotential or multipotential cells prior to irreversible commitment. In this context comparisons with other receptor-mediated responses will be useful. Some hormonal ligands (eg, insulin, vasopressin) not only regulate the level of their complementary receptors in target cells (and hence the cells' intrinsic sensitivity to a repeat stimulus) but can concomitantly induce the transmodulation of receptors for other ligands on the same cell.[134] This may provide a general mechanism for preempting a competing pathway of cell regulation. It is relevant also to note that lineage choice can be regulated or directed by external signals in other cellular systems involving bipotential cells (in vitro), eg, astrocyte *v* oligodendrocyte differentiation in glial progenitor cells[135] and cholinergic *v* adrenergic differentiation in ganglion cells.[136]

These issues will be more amenable to direct experimentation when multipotential stem cells retaining differentiation capacity can be selected, cloned, and manipulated more effectively in vitro. It may soon be possible, however, to test the validity of the limited promiscuity idea, some predictions of which might include the following: (1) Clonogenic cells in some cases of acute leukemia should coexpress early markers of more than one hematopoietic lineage in contrast to their more mature descendants and nonhematopoietic tumor cells, which should not. (2) Some normal, multipotential stem cells should coexpress at detectable levels some early differentiation-associated antigens or growth factor receptors of more than one lineage (eg, CD-7 plus GM-CSF receptors in various combinations) but not markers associated with later maturational stages within particular lineages (eg, CD-1/T6 plus MY9 or glycophorin A). Additionally, at least a fraction of multipotential progenitors should produce abbreviated transcripts of immunoglobulin heavy-chain or the T cell receptor β chain (with or without detectable gene rearrangements) or both, and at least a few normal, myeloid lineage progenitors should express TdT. (3) Some relic, at least of abortive or partial DNA rearrangements of immunoglobulin (DJ_H) or T cell receptor chain (DJ) genes, should be detectable in mature hematopoietic cells (lymphoid and myeloid) of the inappropriate type. These rearrangements should not include heavy-chain V region genes or light-chain genes. (4) Some cells expressing early markers of two lineages should retain the capacity to adopt either lineage.

Clearly, such experiments will require judicious choice of markers and growth factor protein produced from cloned DNA as well as purified progenitors and highly efficient clonal assays.

NOTE ADDED IN PROOF

Two other groups have recently reported clonal rearrangement of Ti β genes in some B cell lineage leukemias: O'Conor et al, Lancet 1:1295, 1985, and Pelicci et al, J Exp Med 162:1015, 1985.

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Part of the Activating Cross-Linked Immunoglobulin G Is Internalized by Human Platelets to Sites Not Accessible for Enzymatic Digestion

By Martin O. Spycher and Urs E. Nydegger

The differential uptake of tritium-labeled immunoglobulin G (IgG) cross-linked with bisdiazonium-benzidine (BDB) (^{3}H-BDB-IgG) by washed, pooled human platelets to sites inaccessible to pronase digestion was tested. Up to 52% of the ^{3}H-BDB-IgG associated with platelets at 37 °C resisted pronase treatment, whereas only 23% of the cross-linked IgG associated with platelets at 4 °C, or at 37 °C but in the presence of deoxyglucose/antimycin A, remained refractory to pronase. This effect was not due to platelet agglutination. Pronase resistance reached a maximum after a 60-minute incubation period at 37 °C. With increasing ^{3}H-BDB-IgG input, both the total cross-linked IgG associated with platelets and the fraction resistant to pronase digestion approached saturation at 4 °C, but not at 37 °C. The proportion of ^{3}H-BDB-IgG bound to platelets at 4 °C that was resistant to pronase treatment increased by 13% within five minutes of warming the platelets to 37 °C. Pretreatment of platelets with 10 mmol/L acetylsalicylic acid (or 10 μmol/L prostaglandin E_1) prior to the addition of ^{3}H-BDB-IgG led to a 74% (95%) inhibition of the ^{3}H-BDB-IgG-induced ^{14}C-serotonin release, but to only a 44% (49%) inhibition of pronase-digestible bound ligand. In contrast, pretreatment with 10 μmol/L cytochalasin B led to a mere 17% reduction of ^{14}C-serotonin release, whereas acquisition of resistance to pronase digestion by the bound ^{3}H-BDB-IgG was inhibited by 90%. Incubation of platelets at 37°C with ^{3}H-BDB-IgG and removal of unbound material prior to the addition of prostaglandin E_1 or deoxyglucose/antimycin A had little effect on the susceptibility of platelet-associated ^{3}H-BDB-IgG to pronase, whereas the addition of cytochalasin B to ^{3}H-BDB-IgG-treated platelets resulted in greatly increased susceptibility of the platelet-associated ligand to pronase. Thus, after binding, ^{3}H-BDB-IgG becomes transferred in an energy-dependent process to pronase-resistant cellular sites, most likely to the open canalicular system.

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THE INCREASED ASSOCIATION of immunoglobulin G (IgG) with human platelets in such clinical disorders affecting platelets as idiopathic thrombocytopenic purpura[1,2] or some cases of systemic lupus erythematosus,[3] myeloproliferative disease,[3] septicemia,[4] Hodgkin's disease,[5] and malignant tumors[6] is now well documented. Current techniques cannot distinguish whether platelet-associated IgG is present in the form of platelet-directed alloantibodies or autoantibodies or as bound immune complexes. In addition, it has been reported that platelets contain intracellular IgG because higher amounts of IgG can be detected in lysates of platelets than on the intact cell.[7] This may be the main reason why methods that use intact rather than lysed platelets for measuring platelet-associated IgG generally result in lower values of platelet-associated IgG.[8–11]

Complexed IgG activates washed human platelets by binding to a putative Fc receptor. This was suggested from the finding that complexed Fc fragments of IgG, but not immune complexes containing F(ab')$_2$ fragments of antibody-activated platelets,[12,13] and only monomeric IgG and Fc fragments thereof, but not F(ab')$_2$ fragments, inhibited activation of the platelets induced by complexed IgG.[13–15]

Initial studies of the binding of bisdiazonium-benzidine (BDB)-cross-linked IgG (BDB-IgG), a potent activator of human platelets, or monomeric IgG to washed human platelets revealed binding that was not saturable and was mostly irreversible.[14–16] Saturable and reversible binding has been shown to platelets of dimeric or higher-order polymeric IgG[1] aggregates at 4 °C[17] or of BDB-IgG or monomeric IgG in the presence of deoxyglucose and antimycin A at 37 °C.[16] This suggested that mere receptor-ligand binding can only be studied on metabolically inhibited platelets and that IgG could be internalized by active platelets at 37 °C. We studied the manner by which BDB-IgG or monomeric IgG were handled by washed, pooled human platelets by making use of various inhibitors of platelet function and studying differential accessibility of noninternalized *v* internalized IgG to pronase.

MATERIALS AND METHODS

Buffers. Buffer A consisted of 90 mmol/L Tris- (hydroxymethyl) aminomethane (Tris), 30 mmol/L NaCl, 10 mmol/L EDTA, and 6.25 mg/mL bovine serum albumin (BSA), pH 7.6. Buffer B consisted of 46 mmol/L Tris, 7.5 mmol/L *N*-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), 15.5 mmol/L KCl, 78 mmol/L NaCl, 5 mmol/L EDTA, and 3.13 mg BSA, pH 7.4.

Platelet preparations. Platelet-rich plasma was prepared from citrated blood as previously described.[18] Two parts of platelet-rich plasma were mixed with one part of 30 mmol/L citric acid, 3 mmol/L KCl, 102 mmol/L NaCl, and 4.8 mmol/L glucose, pH 6 5, after adjustment of the pH to 6.5 Thereafter, platelet-rich plasma was incubated at 37 °C for 30 minutes, and platelets were washed three times by repeated centrifugation for ten minutes at 1,200 g at room temperature and resuspension in washing buffer containing 3 mmol/L 2(*N*-morpholino) ethane sulfonic acid (MES), 3 mmol/L KCl, 147 mmol/L NaCl, and 4 8 mmol/L glucose, pH 6.5. Finally, they were resuspended in 28 mmol/L TES, 3 mmol/L KCl, 108 mmol/L NaCl, and 25 mmol/L glucose, pH 6.8, at a concentration of 5.1 × 10^9/mL and incubated for one hour at room temperature. In experiments with deoxyglucose- and antimycin A–treated platelets, glucose was omitted in the third washing step, and 25 mmol/L deoxyglucose and 1 μmol/L antimycin A instead of 25 mmol/L glucose were included in the final resuspension buffer.

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Protein Preparations and Labeling With Tritium. The IgG was obtained by cold ethanol fractionation and contained IgG:IgM:IgA in a ratio of 600:8:1. It was cross-linked with BDB according to Pfueller et al.[14] To remove IgG not cross-linked and smaller aggregates, BDB-IgG was gel filtered on a Biogel A-5m column (Bio-Rad Laboratories; Richmond, Va), and only protein eluted in the void volume consisting of aggregates with ten to 30 IgG molecules[14] was used. BDB-IgG, monomeric IgG (IgG obtained from the upper half of the contents in a test tube after centrifugation at 100,000 g for one hour at 4 °C), and human serum albumin were labeled with ^{3}H by reductive methylation as described.[19] In brief, 3 to 4 mg of protein in 500 μL 10 mmol/L Na-phosphate and 0.14 mol/L NaCl, pH 7.0, were mixed at 4 °C with 800 μL of 0.5 mol/L Na-diethylborate, pH 9.0, and 155 μL of fresh 20 mmol/L formaldehyde, and 30 seconds later, 100 μL of ^{3}H-NaBH$_4$ (approximately equal to 5 mCi in 10 mmol/L NaOH, 8.8 Ci/mmol/L; New England Nuclear, Boston) were added. After 20 minutes at 4 °C, 20 μL of 0.1 mol/L NaBH$_4$ was included, and after a further 20 minutes, 20 μL of glycerine was added and the pH adjusted to about 7 with 20% acetic acid. After exhaustive dialysis against 10 mmol/L Tris-HCl and 0.14 mol/L NaCl, pH 8.0, labeled protein was stored at −70 °C in aliquots. Specific activity was within the range 0.8 to 1.8×10^5 cpm/μg.

Measurement of labeled protein associated with washed platelets and pronase treatment. Before use, ^{3}H-BDB-IgG and BDB-IgG were centrifuged for ten minutes at 4 °C and 40,000 g to remove insoluble material, and monomeric IgG preparations were centrifuged at 100,000 g at 4 °C for one hour, whereupon only the upper half of the supernatant was used to eliminate self-aggregated IgG as far as possible. Four parts buffer A were incubated at 37 °C or 4 °C with two parts washed platelets (5.1×10^9/mL) and one part 0.15 mol/L NaCl with slow stirring. After 5 minutes, one part labeled protein was added and incubation continued with slow stirring for 30 minutes at the temperature indicated. Then all tubes were cooled to 4 °C and centrifuged at this temperature for 15 minutes at 2,000 g. The supernatants were discarded and the sediments resuspended with eight parts cold buffer B Duplicate 600-μL aliquots of each sample were incubated either with 200 μL BSA (20 mg/mL in 0 15 mol/L NaCl) or with 200 μL pronase (20 mg/ml in 0.15 mol/L NaCl, 77 PUK [enzyme activity liberating folin positive amino acids] per mg; Sigma Chemical Co, St Louis). Digestion of surface-bound ligand was allowed to proceed usually for four hours at 4 °C, a time lag considered to be optimal because incubation for a further five hours led only to a 4% decrease in remaining platelet-associated radioactivity and because incubation of platelets with pronase for longer time periods (16 hours at 4 °C) resulted in some platelet damage as determined by the release of ^{14}C-serotonin and ^{3}H-adenine. After incubation of the platelets with pronase and BSA, 150 μL of a mixture of dinonylphthalate:dibutylphthalate (1:2.56) were added, and the tubes were centrifuged at 11,000 g for 30 seconds, separating the platelets from the water phase through the phthalate mixture. Aliquots of the supernatants were counted and the tubes inverted. After removing traces of supernatant using absorbent paper, 150 μL 0.5% Triton X-100 (Bio-Rad, Richmond, Calif) and two drops of xylol were added to the sediments before sonification with a Branson sonifier (Branson Sonic Co, Danbury, Conn) Solubilized platelets were transferred to a counting vial and radioactivity measured in a Packard TriCarb 2450 Liquid Scintillation Spectrometer (Downers Grove, Ill). Inhibitors of platelet activation by ^{3}H-BDB-IgG were added to the incubation mixture instead of the one part 0.15 mol/L NaCl. Experiments were always done in duplicate, which usually did not differ from one another by more than 5%.

Calculations. Normally the amount of platelet-associated ligand remaining after BSA or pronase treatment was calculated first by using the specific activity of the labeled compound. The percentage of pronase-resistant protein was then calculated as 100 times the ratio between the platelet-associated protein in the presence of pronase and in the presence of BSA. In the initial experiments, 200 μg/mL of inulin and 43 μCi of ^{14}C-hydroxymethylinulin (30 μCi/mL, 2.14 μCi/mg; Amersham, England) were included in buffer B to correct for labeled protein trapped within the platelet sediment but not associated with the platelets themselves Calculations were made as previously described[14] These corrections were found to be irrelevant, and therefore the inulin preparations were omitted in later experiments.

Measurement of ^{14}C-serotonin release by washed platelets Platelets were labeled with ^{14}C-serotonin (5-hydroxy (side chain-2-^{14}C)-tryptamine creatinine sulfate, 55 mCi/mmol/L [Amersham, England]) by incubation with the labeled compound at a concentration of 125 nCi/mL of platelet suspension for one hour at room temperature. On an average, 92% of the ^{14}C-serotonin was taken up by the platelets. To measure the release of ^{14}C-serotonin induced by ^{3}H-BDB-IgG, incubations were done in the same way as for measurement of ^{3}H-BDB-IgG association, but imipramine (2 μmol/L final concentration) was added to prevent reuptake of released ^{14}C-serotonin. After the indicated incubation period at 37 °C or 4 °C, 400 μL of the mixture was centrifuged at 11,000 g for 30 seconds, and 200 μL of supernatant was added to 2 mL of scintillator to be counted then in the Packard liquid scintillation spectrometer. The formula for the calculation is as follows: ^{14}C-serotonin released (%) = $[(X - B)/(T - B)] \times 100$, where X is the cpm of the sample after centrifugation, T is the total cpm contained in 200 μL before centrifugation, and B is the control—cpm of 200 μL of supernatant after centrifugation but without ^{3}H-BDB-IgG.

Other methods. (1) Platelets were counted in a TOA platelet counter (TOA, Medical Electronics Co, Kobe, Japan) according to the instructions of the manufacturer. (2) To measure possible degraded products of platelet-associated ^{3}H-BDB-IgG, platelets incubated with ^{3}H-BDB-IgG at 37 °C and 4 °C for 30 minutes were solubilized with 1% sodium dodecyl sulfate (SDS; Bio-Rad, Richmond, Calif), one aliquot was immediately counted, and trichloroacetic acid was added to the other aliquot (10% final concentration). After a four-hour incubation period at 4 °C, the samples were centrifuged, and radioactivity in the supernatants was determined.(3) Slab gel electrophoresis of SDS-solubilized and reduced platelets previously incubated with ^{3}H-BDB-IgG at 4 °C or 37 °C in a 5% to 15% gel was done according to Laemmli,[20] and fluorography of the gels was performed as previously described.[19] (4) Prostaglandin E and cytochalasin B were obtained from Sigma Chemical Co, St Louis. They were dissolved in dimethylsulfoxide to a concentration of 10 mmol/L and stored at −20 °C. Dilutions were made with 0.15 mmol/L NaCl. All other substances were of reagent grade. (5) Protein concentrations were determined with a modified Lowry method as described[15] using BSA as a standard.

RESULTS

Pronase susceptibility of ^{3}H-BDB-IgG or ^{3}H-monomeric IgG (^{3}H-mIgG) associated with washed human platelets at 4 °C or 37 °C. In a first step, the capacity of platelets to take up ^{3}H-BDB-IgG was evaluated under three different conditions: at 37 °C, at 4 °C, and at 37 °C after treatment with deoxyglucose/antimycin A. Figure 1A shows that platelets were able to take up more ^{3}H-BDB-IgG at 37 °C than at 4 °C (0.60 μg compared to 0.38 μg), and in the presence of deoxyglucose/antimycin A, association at 37 °C was reduced to 0.20 μg.

The capacity of pronase to remove platelet-associated ^{3}H-BDB-IgG was also assessed: 52% of the ^{3}H-BDB-IgG

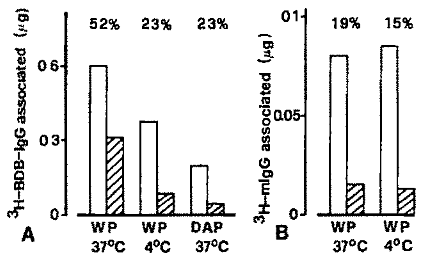

Fig 1. (A) Internalization of ^{3}H-BDB-IgG by washed human platelets as determined by the loss of accessibility to pronase. Washed platelets (WP) or deoxyglucose/antimycin A–treated platelets (DAP) were incubated with ^{3}H-BDB-IgG (12 µg/mL) at 37 °C or 4 °C for 30 minutes. After washing at 4 °C and resuspension in buffer without ^{3}H-BDB-IgG, the platelets were divided into two fractions. To the first fraction 5 mg/mL pronase was added (shaded bars), to the second, 5 mg/mL BSA (open bars). After incubation for six hours at 4 °C, platelets were centrifuged through oil and the radioactivity in the sediment was determined. The ordinate represents ^{3}H-BDB-IgG associated to 7.5 × 10^8 platelets. At the top of each pair of bars, percentages of pronase-resistant ^{3}H-BDB-IgG associated to the platelets are indicated (determined as the ratio between ^{3}H-BDB-IgG associated to pronase- and BSA-treated platelets). (B) The same experiment as in Fig 1A, but washed platelets were incubated with ^{3}H-mIgG (30 µg/mL) instead of ^{3}H-BDB-IgG.

associated with platelets at 37 °C resisted subsequent pronase treatment, whereas only 23% of the ^{3}H-BDB-IgG associated to platelets at 4 °C or to deoxyglucose/antimycin A–treated platelets at 37 °C was refractory to pronase digestion (Fig 1A). Thus, at 37 °C, a 29% increase of pronase-resistant protein was found. When platelets were incubated with ^{3}H-BDB-IgG for 30 minutes at 37 °C, washed, treated with deoxyglucose/antimycin A at room temperature for one hour, and then further incubated at 37 °C for 30 minutes, they still carried 70% of the initial pronase-resistant ^{3}H-BDB-IgG. Thus, the deoxyglucose/antimycin A treatment was unable to make substantial amounts of pronase resistant ^{3}H-BDB-IgG available to pronase digestion. When experiments were carried out with ^{3}H-monomeric IgG (^{3}H-mIgG) instead of ^{3}H-BDB-IgG, they revealed that incubation at either 37 °C or 4 °C allowed similar amounts of ^{3}H-mIgG to become associated with platelets in a way that was highly susceptible to pronase treatment at both temperatures (Fig 1B). To exclude self-aggregated IgG in the mIgG preparation as much as possible, the IgG preparations were always centrifuged immediately before use for one hour at 100,000 g and 4 °C, and only the upper half of the supernatants was used.

Although all experiments were done in the presence of 5 mmol/L EDTA, which is known to prevent platelet aggregation, we investigated whether the decreased pronase susceptibility of ^{3}H-BDB-IgG associated to platelets at 37 °C was possibly due to the entrapment of protein within microscopic aggregates or agglutinates. In fact, pronase resistance of labeled, platelet-associated mIgG was as low as indicated in Fig 1B whether or not 20 µg/mL BDB-IgG was included in the samples during the 37 °C incubation period to activate the platelets, and the same result was obtained using labeled human serum albumin instead of ^{3}H-mIgG (data not shown). This is incompatible with platelet clumping as the major reason for the increased pronase resistance of platelet-associated ^{3}H-BDB-IgG. Nevertheless, platelet activation produced by incremental amounts of ^{3}H-BDB-IgG resulted in some microscopic aggregation or agglutination even in the presence of EDTA as seen from a decrease in platelet numbers determined by electronic counting (Fig 2). However, comparison of aggregation to pronase susceptibility of platelet-associated ^{3}H-BDB-IgG revealed no correlation: 50% pronase-resistant platelet-associated ^{3}H-BDB-IgG was observed with 5 µg/mL of ^{3}H-BDB-IgG, whereas the reduction of platelet count at this concentration was only 2% (Fig 2). In addition, pronase resistance was readily saturable, whereas the reduction in platelet count progressively increased with increasing ^{3}H-BDB-IgG inputs.

The time course of the acquisition of resistance to pronase by ^{3}H-BDB-IgG associated with platelets at 37 °C and at 4 °C is shown in Fig 3. It appears that pronase resistance becomes detectable after two minutes and reaches a plateau after one hour, whereas at 4 °C, pronase was always able to remove the bulk of platelet-associated radioactivity. The same saturation characteristics were obtained when the time course of the ^{3}H-BDB-IgG association with platelets was examined after BSA or pronase treatment (data not shown).

Concentration dependence of ^{3}H-BDB-IgG binding to platelets and its susceptibility to pronase digestion. Increasing amounts of ^{3}H-BDB-IgG were incubated with washed platelets at 37 ° C or at 4 °C for 30 minutes in the presence or absence of 5 mg/mL unlabeled mIgG. Then the amount of ^{3}H-BDB-IgG associated with the platelets after the four-hour incubation with pronase or BSA at 4 °C was determined. Specific binding is referred to as the difference of the amounts of ^{3}H-BDB-IgG found associated to platelets with and without unlabeled mIgG in the system. In parallel tubes, release of ^{14}C-serotonin was measured at 37 °C (Fig 4). As can be seen, specific ^{3}H-BDB-IgG binding approached saturation at 4 °C but not at 37 °C with or without pronase treatment. Significant binding had already occurred at a very low ^{3}H-BDB-IgG concentration, which did not result in serotonin release at 37 °C. Nonspecific binding was substan-

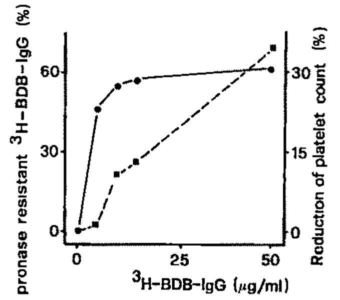

Fig 2. Comparison of the pronase resistance of platelet-associated ^{3}H-BDB-IgG with platelet agglutination or aggregation. Washed platelets were incubated with increasing amounts of ^{3}H-BDB-IgG for 30 minutes at 37 °C. After washing at 4 °C, platelets were counted on a TOA platelet counter, and pronase-resistant platelet-associated ^{3}H-BDB-IgG was determined as described in Materials and Methods. ●——●, Pronase resistance of associated C^{3}H-BDB-IgG; ■----■, reduction of platelet count.

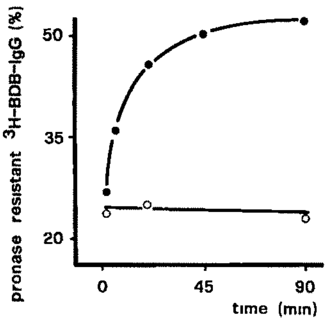

Fig 3. Time course of pronase-resistant platelet-associated ^{3}H-BDB-IgG. ^{3}H-BDB-IgG (10 μg/mL) was incubated with washed platelets at 37 °C (●) or 4 °C (○). At the indicated times, aliquots were taken and kept on ice. After the last aliquot had been taken, 5 mg/mL pronase was added to one half of each aliquot and 5 mg/mL BSA to the other, and further processing was done as described in Materials and Methods.

Table 1. Comparison of the Percentage of Pronase-Resistant ^{3}H-BDB-IgG Specifically Associated With Washed Platelets and Serotonin Release in Relation to Increasing ^{3}H-BDB-IgG Additions to Washed Platelets at 37 °C and 4 °C

^{3}H-BDB-IgG (μg/mL)	Percentage of Pronase-Resistant ^{3}H-BDB-IgG Specifically Associated With Washed Platelets at 37 °C*	4 °C	Percentage of ^{14}C-Serotonin Release at 37 °C†
4	46 (23)	23	1
13 5	54 (28)	26	10
50	77 (46)	31	38
150	72 (41)	31	49

*Experimental procedures as described in Materials and Methods. Values in brackets represent the difference of values at 37 °C and 4 °C

†Platelets incubated with ^{3}H-BDB-IgG at 4 °C did not release ^{14}C-serotonin

tial with high–^{3}H-BDB-IgG input and reached 50% of the total binding at 150 μg/mL of ^{3}H-BDB-IgG. The percentages of pronase-resistant ^{3}H-BDB-IgG specifically associated with washed platelets at 37 °C or at 4 °C are compared in Table 1 with the percentage of ^{14}C-serotonin release over the same range of ^{3}H-BDB-IgG input. It appears that at 37 °C the specific platelet-associated pronase-resistant ^{3}H-BDB-IgG as well as the serotonin release increased depending on the dose of the ^{3}H-BDB-IgG input, whereas at 4 °C, only 8% additional pronase resistance was seen with the highest input of ligand.

When platelets were treated with 100 μg/mL ^{3}H-BDB-IgG at 4 °C for 60 minutes, washed, and warmed to 37 °C for a further five- or 30-minute incubation, pronase susceptibility after both 37 °C incubation periods was decreased by 13% as compared to such susceptibility measured after the 4 °C time period. At the same time, 42% of the ^{14}C-serotonin was released by the platelets. Lower ^{3}H-BDB-IgG inputs (20 μg/mL or 50 μg/mL) resulted in no significant decrease of pronase susceptibility of associated protein whereas ^{14}C-serotonin release was below 18%.

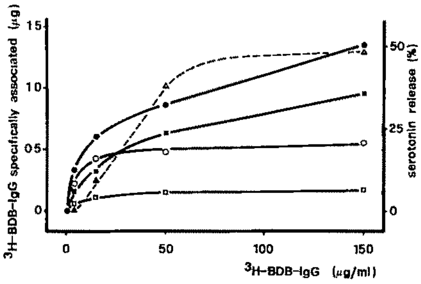

Fig 4. Dose dependence of specific ^{3}H-BDB-IgG association with washed platelets after BSA or pronase treatment (——) and simultaneous measurement of serotonin release. (---) Washed platelets were incubated with increasing amounts of ^{3}H-BDB-IgG for 30 minutes at 37 °C or 4 °C in the presence or absence of 5 mg/mL mIgG, and the amount of ^{3}H-BDB-IgG specifically associated with 7.5 × 10^8 platelets was determined. Specific association represents the difference of association measured in the presence and absence of mIgG. Key: ●, platelets incubated at 37 °C before the addition of BSA; ■, platelets incubated at 37 °C before the addition of pronase; ○, platelets incubated at 4 °C before the addition of BSA; and □, platelets incubated at 4 °C before the addition of pronase. The ^{3}H-BDB-IgG–induced serotonin release at 37 °C (from platelets prelabeled with ^{14}C-serotonin) was measured (△) in parallel tubes.

Inhibition of ^{3}H-BDB-IgG association with platelets by monomeric IgG and by reagents interfering with platelet activity at different levels. Inhibition of the ^{3}H-BDB-IgG association with washed platelets at 37 °C or 4 °C induced by incremental amounts of mIgG is shown in Fig 5. For these experiments, mIgG was added to the platelets five minutes before ^{3}H-BDB-IgG, and it can be seen that mIgG inhibits the uptake of ^{3}H-BDB-IgG by the platelets at both incubation temperatures independently whether or not pronase was added. Pronase resistance of platelet-associated ^{3}H-BDB-IgG was also inhibited by mIgG, inhibition of the ^{3}H-BDB-IgG association occurring at lower mIgG inputs than inhibition of pronase resistance (data not shown).

The effects of acetylsalicylic acid (which interferes with the prostaglandin pathway by inhibiting cyclooxygenase[21]), prostaglandin E$_1$ (which elevates the intracellular cAMP concentration[21]) and cytochalasin B (which interacts with actin[22]) on pronase susceptibility of platelet-associated ^{3}H-

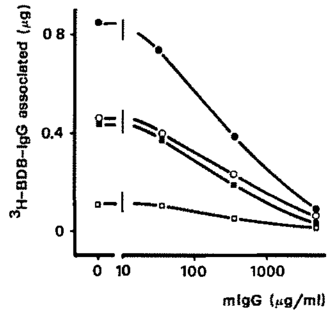

Fig 5. Inhibition by mIgG of ^{3}H-BDB-IgG association with washed platelets. Washed platelets were incubated with increasing amounts of mIgG for five minutes at 37 °C or 4 °C before the addition of ^{3}H-BDB-IgG (15 μg/mL). After a further incubation for 30 minutes at the corresponding temperature, platelet-associated protein was determined as described in Materials and Methods. For symbols see Fig 4.

BDB-IgG were studied. In a first set of experiments, washed platelets were incubated with the inhibitor for five minutes at 37 °C before the addition of ^{3}H-BDB-IgG and a further incubation for 30 minutes at 37 °C. Then the percentage of pronase-resistant, platelet-associated ^{3}H-BDB-IgG was determined. The same experiment was also conducted at 4 °C. At this temperature, at which platelets are not activated by ^{3}H-BDB-IgG, the percentage of pronase-resistant protein did not change significantly and was within a range of 18% to 23% whether or not inhibitors were present. Table 2, column 2, shows the inhibition of the increased pronase resistance of platelet-associated ^{3}H-BDB-IgG induced during the activation of the platelets at 37 °C. In parallel tubes, the ^{3}H-BDB-IgG–induced release of ^{14}C-serotonin at 37 °C was also measured (Table 2, column 4). Although acetylsalicylic acid and prostaglandin E_1 had a marked inhibitory effect on ^{3}H-BDB-IgG–induced serotonin release, their effect on preventing pronase from removing ^{3}H-BDB-IgG associated to platelets was less distinct. In contrast, addition of cytochalasin B led to an increased protease susceptibility of platelet-associated ^{3}H-BDB-IgG but had little or no inhibitory effect on ^{3}H-BDB-IgG–induced serotonin release. In a second set of experiments, platelets were incubated with ^{3}H-BDB-IgG for 30 minutes, one sample at 37 °C, the other at 4 °C; both samples were washed at 4 °C and incubated thereafter with inhibitor at the corresponding temperature for 30 minutes (Table 2, column 3). It was found that 10 μmol/L prostaglandin E_1 had no effect on the pronase resistance of platelet-associated ^{3}H-BDB-IgG, whereas increasing cytochalasin B input led to an inhibition of the increased pronase resistance of platelet-associated ^{3}H-BDB-IgG obtained at 37 °C, the extent being not so marked as the decrease observed upon simultaneous incubation of cytochalasin B and ^{3}H-BDB-IgG with platelets. Again, both inhibitors had no effect on the pronase resistance of ^{3}H-BDB-IgG associated with platelets at 4 °C.

Table 2. Effect of Inhibitors of Platelet Functions on the Resistance to Pronase of Platelet-Associated ^{3}H-BDB-IgG and the Induction of Serotonin Release by ^{3}H-BDB-IgG

	Inhibition of Pronase Resistance (%)*		
Inhibitor	By Simultaneous Incubation of Inhibitor and ^{3}H-BDB-IgG With Platelets†	By Incubation of Inhibitors With Platelets Pretreated With ^{3}H-BDB-IgG‡	Inhibition of ^{14}C-Serotonin Release Induced by ^{3}H-BDB-IgG at 37 °C (%)†
Acetylsalicylic acid			
1.0 mmol/L	19	ND	50
10.0 mmol/L	44	ND	74
Prostaglandin E_1			
0.2 μmol/L	15	ND	46
1.0 μmol/L	24	ND	74
10.0 μmol/L	49	0	95
Cytochalasin B			
0.2 μmol/L	11	0	1
0.5 μmol/L	40	16	ND
1.0 μmol/L	60	31	10
10.0 μmol/L	90	77	17

Abbreviation: ND, not determined.

*Numbers represent the percent inhibition of the increased pronase resistance of ^{3}H-BDB-IgG associated with platelets at 37 °C. This is calculated as 100 times the ratio between the difference of pronase resistant ^{3}H-BDB-IgG from samples incubated at 37 °C and 4 °C in presence of the inhibitor and the same difference without the inhibitor.

†Washed platelets were incubated with the inhibitor for five minutes before the addition of ^{3}H-BDB-IgG (30 μg/mL) and a further 30-minute incubation. Two samples were run, one at 37 °C, the other at 4 °C. Release of ^{14}C-serotonin induced by 30 μg/mL ^{3}H-BDB-IgG at 37 °C was assessed in parallel tubes. At 4 °C there was no release of ^{14}C-serotonin.

‡Washed platlets were incubated with ^{3}H-BDB-IgG at either 37 °C or 4 °C for 30 minutes, washed at 4 °C, and resuspended in buffer B before the addition of inhibitors and a further 30-minute incubation at the corresponding temperature.

Finally, we were interested to know whether platelet-associated ^{3}H-BDB-IgG is processed further by intrinsic platelet enzymes. For this purpose, ^{3}H-BDB-IgG was incubated with platelets at 37 °C and 4 °C for 30 minutes or 120 minutes, respectively; then the platelets were solubilized with SDS, and trichloroacetic acid–precipitable radioactivity was measured: no decrease in precipitable radioactivity was found. In addition, gel electrophoresis of ^{3}H-BDB-IgG obtained from the solubilized platelets and subsequent fluorography of the gel showed no degradation of the aggregated IgG into smaller components.

DISCUSSION

We demonstrate that part of aggregated immunoglobulin used for the activation of platelets becomes inaccessible to prolonged pronase digestion probably because of displacement to intracellular sites. Our findings may explain the observation that saturable and reversible association of aggregated IgG with washed human platelets can be obtained at 4 °C[17] but can only be demonstrated at 37 °C in the presence of metabolic inhibitors deoxyglucose and antimycin A.[16] Thus, evidence is provided that the engulfment of aggregated IgG taking place at 37 °C is energy dependent and does not result from either passive diffusion to pronase-inaccessible sites or entrapment within platelet aggregates or agglutinates. This is based on the following arguments: (1) EDTA (5 mmol/L), which is known to prevent platelet aggregation, was always included in the reaction mixtures. (2) Although with the addition of incremental amounts of ^{3}H-BDB-IgG to constant amounts of platelets some decrease in the platelet count occurred in the reaction tubes (Fig 2), a simultaneous evaluation of pronase resistance revealed an increase of engulfed material that already occurred at much lower ^{3}H-BDB-IgG inputs and then plateaued. In addition, the day-to-day variation in the platelet count decrease was substantial during these experiments, whereas pronase resistance remained constant within small limits. (3) There was no change in the proportion of platelet-associated labeled mIgG or human serum albumin that was resistant to pronase digestion after the addition of 20 μg/mL BDB-IgG, a concentration of BDB-IgG known to activate the platelets even in the presence of small amounts of ^{3}H-mIgG or human serum albumin. (4) The omission of stirring during the incubation of ^{3}H-BDB-IgG with the platelets did not lead to a decreased pronase resistance of platelet-associated ^{3}H-BDB-IgG (data not shown).

If such a reduction of pronase-accessible surface area by aggregation or agglutination of platelets is not the reason why part of the ^{3}H-BDB-IgG associated at 37 °C escapes pronase digestion, then the assumption appears justified that the binding of ^{3}H-BDB-IgG to the platelet plasma membrane is followed by redistribution to an area not accessible to pronase digestion. There is a difference in the extent of the ^{3}H-BDB-IgG associated with platelets and its pronase resistance depending on whether the platelets were incubated with the IgG aggregates at 37 °C or preincubated with the aggregates at 4 °C and then warmed to 37 °C after the removal of unbound material. When incubation was at 4 °C prior to warming to 37 °C, the fraction of ^{3}H-BDB-IgG that was not susceptible to pronase digestion was only increased by 13% even at the highest input of IgG aggregates (100 μg/mL), with no significant increase at lower inputs of ^{3}H-BDB-IgG. In contrast, incubation of the platelets at 37 °C with low amounts of ^{3}H-BDB-IgG (20 μg/mL) resulted in a 29% increase of pronase-resistant platelet-associated ^{3}H-BDB-IgG. Simultaneous measurement of the ^{14}C-serotonin release by platelets treated with ^{3}H-BDB-IgG at 4 °C and then warmed up revealed a reduced release compared to that measured by the incubation of ^{3}H-BDB-IgG with washed platelets at 37 °C, although the extent of reduction was not so pronounced as compared to the reduction of pronase resistance. It appears that during centrifugation after the 4 °C incubation period a considerable part of the ^{3}H-BDB-IgG is lost from its binding sites, consistent with earlier findings in which it was shown that most of reversibly bound IgG aggregates dissociate from the membrane within minutes,[14,16,17] although binding at 4 °C to platelets is essentially complete after a 60-minute incubation period (data not shown).[17] In addition, it cannot be excluded that the binding of aggregates to sites from where they could redistribute at 37 °C is temperature dependent and could be diminished at 4 °C. Nevertheless, both serotonin release and the limited increase of the pronase resistance of associated ^{3}H-BDB-IgG occurred immediately, within five minutes after warming to 37 °C, consistent with a receptor-mediated process in which the binding of the ligand is the rate-limiting step.[23] This obviously was the case in our experiments because incubation of ^{3}H-BDB-IgG with platelets at 37 °C resulted in a maximal increase of pronase-resistant platelet-associated ^{3}H-BDB-IgG at much later stages of the incubation period (Fig 3). Moreover, high amounts of mIgG inhibited both the total association of ^{3}H-BDB-IgG to washed platelets and the pronase-resistant part of it (Fig 5).

Because pronase resistance of ^{3}H-mIgG was not influenced by the presence of BDB-IgG, eg, on activated platelets, receptor clustering through the simultaneous binding of one BDB-IgG molecule to at least two receptors must be envisaged as the triggering mechanism for escape from pronase action. Receptor clustering has been described for lymphocytes,[24,25] whereby two forms are distinguished, the energy-independent patching and the energy-dependent capping that engages the cytoskeleton of the cell. Recently, patching and capping of concanavalin A receptors have also been described on platelets.[26] The capping phenomenon was not dependent on extracellular divalent cations and was inhibited by cytochalasin B, implying that an intact microfilament structure was necessary. Our results obtained with acetylsalicylic acid, prostaglandin E$_1$, and cytochalasin B strongly indicate that the redistribution phenomenon of ^{3}H-BDB-IgG on platelets closely resembles a capping phenomenon. Engulfment of ^{3}H-BDB-IgG was less inhibited by acetylsalicylic acid and prostaglandin E$_1$ than was serotonin release. In contrast to these inhibitors, cytochalasin B, which binds to actin, was able to inhibit engulfment to a greater extent than release of serotonin. We conclude from these results that engulfment of ^{3}H-BDB-IgG depends on a functionally intact cytoskeleton.

Although we have no definitive proof of the site in the platelet to which ^{3}H-BDB-IgG moves to become resistant to pronase digestion, there are strong indications that it is transferred to the open canalicular system. Incubation of platelets pretreated with ^{3}H-BDB-IgG at 37 °C with prostaglandin E$_1$ or deoxyglucose/antimycin A had no or only little effect on pronase resistance, whereas cytochalasin B administrations led to a substantial decrease in pronase-resistant ^{3}H-BDB-IgG (Table 2). This supports the idea that most of the engulfed ^{3}H-BDB-IgG must still be connected to the surface, thus becoming accessible to pronase digestion after disruption of actin filaments. Whether cytochalasin B allows engulfed ^{3}H-BDB-IgG to float back to the surface or whether the opening of the open canalicular system is dilated by the drug cannot be decided from our results. Uptake of proteins into the open canalicular system has been shown by electron microscopy with horseradish peroxidase, cationized ferritin, or ferritin-conjugated wheat germ agglutinin. Peroxidase and cationized ferritin were readily taken up at 37 °C but not at 4 °C,[27] and cationized ferritin or ferritin-conjugated wheat germ agglutinin were taken up at 37 °C into washed platelets but not into washed and fixed platelets.[28,29] Although hardly any pinocytic vesicles were observed,[27] coated pits have been reported.[28,30] Therefore, further processing of engulfed ^{3}H-BDB-IgG is possible, eg, in lysosomes. As yet, we could not demonstrate detectable digestion of ^{3}H-BDB-IgG associated with platelets after two hours at 37 °C, a time long enough to reliably detect digested ligand in phagocytic cells.[31] Whether small amounts of engulfed ^{3}H-BDB-IgG are transferred to storage organelles or whether further engulfment is dependent on extracellular divalent cations needs further investigation.

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Partial Characterization of a Binding Site For von Willebrand Factor on Glycocalicin

By Alan D. Michelson, Joseph Loscalzo, Barbara Melnick, Barry S. Coller, and Robert I. Handin

The binding of von Willebrand factor (vWF) to platelet membrane glycoprotein Ib (GpIb) facilitates platelet adhesion to vascular subendothelium. In this study, we provide evidence that the vWF binding site is on glycocalicin (GC), a proteolytic fragment of GpIb, and we examine the role of the carbohydrate portion of GC on that binding. The binding to platelets of 6D1, a monoclonal antibody that recognizes an epitope on GpIb and blocks ristocetin-induced vWF binding to platelets, was inhibited by purified GC. In addition, purified GC inhibited ristocetin-dependent binding of ^{125}I-labeled vWF to platelets. Since GC contains 60% carbohydrate by weight, we assessed the role of carbohydrate sequences on its interaction with antibody 6D1 and vWF. Based on the known sequence of the major oligosaccharide chain of GC—*N*-acetyl neuraminic acid, galactose, *N*-acetyl glucosamine, *N*-acetyl galactosamine—we treated GC sequentially with neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase. Removal of sialic acid and galactose residues did not affect GC binding. Removal of *N*-acetyl glucosamine residues did not affect GC binding to 6D1 but did decrease the ability of GC to inhibit vWF binding to platelets, increasing the concentration needed to inhibit binding by 50% (IC_{50}) 40-fold. This suggests that a portion of the oligosaccharide chains on GC contributes to the vWF binding activity of this molecule.
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THE VON WILLEBRAND FACTOR (vWF) is a heterogeneous polymeric plasma glycoprotein made up of a series of multimers with estimated molecular masses from 1×10^6 to 20×10^6 daltons.[1] The binding of vWF multimers to platelets facilitates platelet adhesion to vascular subendothelium.[2] Although the stimulus that initiates vWF binding to platelets in vivo is not known, binding can be induced in vitro by incubating platelets with the cationic glycopeptide antibiotic ristocetin.[3] There is considerable evidence that the platelet membrane protein glycoprotein Ib (GpIb) contains one of the binding sites for vWF and that binding to this site is facilitated by ristocetin.[2] There is also evidence that binding of vWF to this site enhances platelet adhesion.[4]

The precise region on GpIb to which vWF binds is unknown, although there is some evidence that it may be localized to a proteolytic fragment of GpIb—glycocalicin (GC). GC has been purified by several laboratories,[5-7] and a monoclonal antibody has been developed that recognizes an epitope on GC, inhibits ristocetin-dependent platelet agglutination, and blocks vWF binding to platelets.[8] In addition, Okumura and Jamieson reported that purified GC inhibited ristocetin-induced platelet agglutination.[9]

Our studies provide additional evidence that the vWF receptor site on GpIb is present on GC and that binding to this site can be studied without the need for detergents to keep GpIb in solution. Although many other cell membrane receptors have been purified and studied in vitro,[10-13] detergents were needed to keep these integral membrane proteins, which are hydrophobic, in solution. It has been possible to study only a few other membrane receptors like the hepatic receptor for IgA[14] and the hepatic asialoglycoprotein receptor[15] in a lipid-free aqueous environment.

These two properties of GC, its solubility in aqueous buffers and its ability to block vWF-dependent platelet reactions, allowed us to quantify vWF binding to GC and to determine some of the structural features of GC that facilitate binding. We have focused on the role of carbohydrate sequences on GC since the molecule is 60% carbohydrate by weight and the sequence of its major oligosaccharide has recently been reported.[16] Although carbohydrate determinants of other receptors may play a role in ligand binding, this functional role can only be inferred from studies on whole cells or partially purified materials. In contrast, with the availability of this purified receptor fragment, we have been able to demonstrate a role for the *N*-acetyl glucosamine residues on GC in the binding of vWF.

MATERIALS AND METHODS

Materials

Polyethylene glycol (PEG), 2-mercaptoethanol (2-ME), iodoacetamide, poly-L-lysine (<M_r>) = 400,000), bovine gamma globulin (Cohn fraction II), bovine albumin (Cohn fraction V), fetuin (type III), and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma Chemical Co, St Louis. Sodium dodecyl sulfate (SDS) and SDS-PAGE High Molecular Weight Standards were purchased from Bio-Rad Laboratories, Richmond, Va. $Na^{125}I$ and a sheep antimouse immunoglobulin $F(ab')_2$ fragment conjugated with horseradish peroxidase were purchased from New England Nuclear, Boston. Dulbecco's modification of Eagle's medium was purchased from Flow Laboratories, McLean, Va. Ristocetin was purchased from Bio-Data Corp, Horsham, Pa. Iodo-beads were purchased from Pierce Chemical Co, Rockford, Ill. Immulon 1 Plates were purchased from Dynatech Laboratories, Inc, Alexandria, Va. Sephacryl S-1000, Sephadex G-25, Sepharose 6B, and Wheat Germ Lectin-Sepharose 6MB were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Neuraminidase (1 IU/mL) purified from *Vibrio cholerae*, *N*-2-hydroxyethylpiperazine-*N*-'2-ethanesulfonic acid

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(HEPES), *o*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl-2-acetamido-2-deoxy-α-D-glucopyranoside were purchased from Calbiochem-Behring Corp, La Jolla, Calif. β-galactosidase (1,500 U/mL) purified from *E coli*, β-*N*-acetyl glucosaminidase (20 U/mL), and galactose dehydrogenase were purchased from Boehringer Mannheim, Indianapolis, Ind. Lactoperoxidase, *N*-acetyl neuraminic acid, galactose, *N*-acetyl glucosamine, and *N*-acetyl galactosamine, all of the highest grade available, were purchased from Sigma Chemical Co, St Louis. Trypsin, treated with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone (TPCK) to inhibit contaminant chymotryptic activity, was purchased from Worthington Diagnostic Systems, Inc, Freehold, NJ. Amicon Diaflo Membrane PM30 was purchased from Amicon Corp, Danvers, Mass. *Serratia* protease was a very generous gift of Dr H. Cooper (University of North Carolina, Chapel Hill). All other materials used were of reagent grade or better, and deionized water was used throughout.

Methods

Platelet counting was performed on a Coulter Counter Model F (Coulter Electronics, Hialeah, Fla) Absorbance was measured in a Gilford Spectrophotometer 2400 (Gilford, Oberlin, Ohio), except in the platelet enzyme-linked immunosorbent assay (ELISA) inhibition assay in which a Bio-Tek EIA Reader (Bio-Tek Instruments, Inc, Burlington, Vt) was used. Gamma emission was assayed using a Beckman Model 8000 Gamma Counter (Beckman Instruments, Inc, Fullerton, Calif). Platelet aggregometry was performed on a Payton Dual Channel Aggregometer (Payton Assoc, Inc, Buffalo) and changes in light transmission recorded with an Omniscribe Recorder (Houston Instruments, Austin, Tex).

Fresh platelets. Fresh platelets used in the vWF binding assay were prepared from 20-mL venous blood drawn into 3-mL acid-citrate-dextrose (ACD). After mixing, the sample was centrifuged at 200 g for 10 minutes at room temperature, and only the upper two thirds of the platelet-rich plasma (PRP) was collected to minimize leukocyte and red cell contamination. The pH of the PRP was adjusted to 6.5 by drop-wise addition of ACD and then centrifuged at 3200 g for 10 minutes at room temperature. The platelet pellet was resuspended in 10 mL of 10 mmol/L Tris, 0.15 mol/L NaCl, 20 mM EDTA, pH 7.4, and the centrifugation and washing repeated five times. An additional two washes were performed under the same conditions, except that the platelet pellet was resuspended in 10 mL of 10 mmol/L tris(hydroxymethyl) aminomethane (Tris), 0.15 mol/L NaCl, pH 7.4 (TBS). The platelets were diluted in TBS to a concentration of 5×10^5 per μL for the vWF binding assay.

Formalin-treated platelets. Formalin-treated platelets[17] used in ristocetin agglutination assays were prepared by mixing an equal volume of 2% paraformaldehyde in TBS with freshly prepared PRP in 14 mmol/L sodium citrate and incubating for one hour at 37 °C. Platelets were then either immediately washed or stored at 4 °C for up to 16 hours, after which they were washed twice with 10 mmol/L Tris, 0.15 mol/L NaCl, 20 mmol/L EDTA, pH 7 4, then twice with TBS. Platelets were resuspended at $4 \times 10^5/\mu$L and stored at 4 °C for up to three weeks.

Glycocalicin purification. A modification of the method described by Solum et al[18] was used. Fifty units of outdated platelets (obtained from the American Red Cross Blood Services, Northeast Region, Boston) were pooled and centrifuged at 3,200 g for 30 minutes at 4 °C. The sedimented platelets were resuspended in wash buffer (0.15 mol/L NaCl, 5 mmol/L glucose, 0.6 mmol/L EDTA, 10 mmol/L Tris, pH 7.6) and then centrifuged at 250 g for 15 minutes to remove red cells. The platelets were then washed by centrifuging twice at 3,200 g for 30 minutes, the first resuspension in wash buffer and the second in 320 mL warm (37 °C) 3 mol/L KCl. After the second resuspension, the platelets were incubated for 30 minutes at 37 °C and then centrifuged at 8,000 g for 10 minutes The supernatant (KCl extract) was dialyzed for 18 hours against several changes of buffer A (0.15 mol/L NaCl, 10 mmol/L Tris, 0.6 mmol/L EDTA, 0.01% NaN_3, pH 7.6) and then loaded onto a 10-mL column of Wheat Germ Lectin-Sepharose 6-MB. The column was washed with buffer A until the absorbance at 280 nm returned to baseline levels. The column was then washed with 2.5% *N*-acetyl glucosamine in buffer A, and peak fractions, as determined by absorbance at 280 nm, were pooled and dialyzed against buffer B (1 mol/L NaCl, 10 mmol/L Tris, 0.6 mmol/L EDTA, 0.01% sodium azide, pH 7.6). This fraction was concentrated to 15 mL by ultrafiltration (Amicon Diaflo Membrane PM 30) and applied to a Sepharose 6B column (2.5 × 90 cm). The fractions showing maximal activity in the platelet ELISA inhibition assay were pooled, dialyzed against distilled water, and lyophilized. The yield was approximately 1 mg protein per preparation, as determined by Lowry's method.[19] Before use in assays, GC was reconstituted in TBS, pH 7.4. Incubation of the GC with 0.6 mol/L 2-ME followed by electrophoresis on a 6% polyacrylamide gel in the presence of SDS demonstrated a single band of an approximate molecular weight of 140,000 daltons after staining of the gel with Coomassie blue and periodic-acid Schiff's (PAS) reagent.[20] GC molarity was calculated from its molecular weight and protein concentration, assuming that GC was 60% carbohydrate on a weight basis.[6] GC concentration was also determined by measurement of the absorbance at 280 nm. The specific absorbance of GC at 280 nm was 17.4 based on the protein concentration determined by Lowry's method, with a correction for the carbohydrate content.

vWF purification. Unless otherwise indicated, all steps were performed in polyethylene containers at 25 °C. Two and one-half liters of plasma, prepared from blood collected into citrate-phosphate-dextrose anticoagulant, were obtained within two to three days of collection from the American Red Cross Blood Services, Northeast Region (Boston) and frozen at −30 °C for 24 hours. After thawing at 4 °C for 24 hours, the cryoprecipitate was collected by centrifugation at 5,000 g for 20 minutes. The pellet was resuspended in 20 mmol/L sodium citrate and the pH adjusted to 6.1 with dropwise addition of 20 mmol/L citric acid. Forty percent PEG (wt/vol) ($\langle M_r \rangle = 8{,}000$) was added to give a final concentration of 5% and the resulting precipitate discarded. Additional PEG was added to 12% and the precipitate collected by centrifugation at 5,000 g for 20 minutes. The pellet was gently rinsed once with 10 mL of 50 mmol/L Tris, 0.15 mol/L NaCl, 0.02% NaN_3, 8% ethanol, pH 7.8, and once with 10 mL of 50 mmol/L Tris, 0 15 mol/L NaCl, and 0.02% NaN_3, pH 7.8 (column buffer). The pellet was warmed to 37 °C, dissolved in 10 mL of column buffer and applied to a siliconized glass column containing Sephacryl S-1000 (95 × 2.5 cm) Four milliliter fractions were collected at a 70 mL/h flow rate

Fractions were analyzed for protein concentration and ability to support ristocetin-dependent platelet agglutination.[21,22] Fractions were stored at 4 °C for up to five days without an appreciable loss of ristocetin cofactor (VIII:RCof) activity. The most active fractions, which had activities of approximately 100 units of ristocetin cofactor activity per mg protein (with the activity of pooled plasma being defined as 1 U/mL), were used in all studies. Protein purity of fractions treated with 0.6 mol/L 2-ME was assayed by SDS gels containing 4% polyacrylamide. There was a single 230,000-dalton band in the most active column fractions. Average protein yields were 3 mg/2.5 L plasma vWF was concentrated, when necessary, by ultracentrifugation at 100,000 g for two hours at 4 °C or by dialysis against 20% (wt/vol) PEG ($\langle M_r \rangle = 20{,}000$) at 4 °C.

Native vWF polymer was reduced for 60 minutes at 22 °C with 50 mmol/L 2-ME and alkylated for 15 minutes at 22 °C with 75 mmol/L iodoacetamide. This limited reduction and alkylation resulted in the production of a dimer of vWF[23] that had the same electrophoretic mobility as unreduced fibronectin ($<M_r> \approx 440,000$) when analyzed on 4% polyacrylamide gels in the presence of SDS. In the binding studies presented here, vWF dimer was used because of its uniform size. This avoids the difficulties encountered in quantifying binding with a heterogeneous series of native vWF multimers.

Radioiodination of vWF Two Iodo-beads were incubated with 0.5 to 1 0 mCi of $Na^{125}I$ in 0.1 N NaOH at 25 °C for 10 minutes. To this was added 1.5 mL 0.1 mg/mL vWF in TBS, and the incubation continued for 25 minutes with gentle rocking. The protein solution was then applied to a 10 × 0.7-cm Sephadex G-25 column that had been previously treated with 0.5 mL of 5 mg/mL bovine serum albumin in TBS. Twelve 0.3-mL fractions were collected and assayed for both total and 25% trichloroacetic acid-precipitable radioactivity. Routinely, column fractions 5 to 7 contained a maximal protein-bound activity of approximately 0.1 μCi/μg. These fractions were 80% to 90% precipitable with trichloracetic acid, and they were equivalent to uniodinated vWF on SDS PAGE and in ristocetin cofactor assays.

^{125}I-vWF/platelet binding assay. Inhibition of vWF binding to fresh platelets by GC was measured by a modification of the vWF/platelet–binding assays of Kao et al[3] and Morisato and Gralnick.[24] The following reagents diluted in TBS were mixed at room temperature: 100 μL of 0.1% bovine serum albumin, 50 μL of 10 mg/mL ristocetin, 20 μL of 5×10^5 fresh platelets per μL varying nanomolar concentrations of GC, a final concentration of 0 48 μg/mL unlabeled and ^{125}I-labeled vWF dimer, and sufficient TBS to bring the total assay volume to 500 μL. Parallel assays to determine nonspecific binding were always performed with the identical reagents without ristocetin. After 30 minutes mixing, 200 μL was removed, gently layered onto 500 μL of 50% sucrose, and centrifuged at 8,700 g for four minutes in a Beckman Microfuge. The two fluid layers were separately aspirated with Pasteur pipettes, with approximately 50 μL sucrose and the pelleted platelets left in the tube. The tubes were then assayed for gamma emission. The ristocetin-dependent binding of vWF to platelets was determined by subtracting the calculated value for vWF bound in the assay without ristocetin from the vWF bound in the corresponding assay with ristocetin. The total vWF bound in the presence of ristocetin was typically five times the value without ristocetin. A 100-fold excess of nonradioactive vWF reduced binding of ^{125}I-labeled vWF to the values seen when ristocetin was omitted from the assay. The binding of vWF to platelets was saturable, with 42 ± 2 ng vWF bound per assay (5×10^6 platelets) at saturation. A final concentration of 0.16 μg/mL vWF resulted in 50% binding. The binding of vWF to platelets for a given GC concentration was expressed as a percentage of the binding of vWF to platelets in the absence of GC.

Platelet ELISA inhibition assay. A monolayer of platelets was fixed in plastic microwells by the following method. Fifty milliliters of venous blood was drawn into 7.5 mL of ACD, PRP prepared, and the platelet pellet washed three times as previously described. The platelets were diluted in TBS, 4 mmol/L EDTA, pH 7.4, to a concentration of $10^5/\mu$L. Poly-L-lysine at a concentration of 10 μg/mL was incubated for 30 minutes at room temperature in microwells of flat-bottomed polystyrene plates. The plates were then flicked to remove the poly-L-lysine, and any remaining drops were aspirated. One hundred microliters of the platelet suspension was added to each microwell and the plates centrifuged at 800 g for five minutes. Without removing the buffer, 50 μL of 0.5% formaldehyde (wt/vol) in TBS, 4 mmol/L EDTA, pH 7.4, was added to each microwell, incubated at room temperature for 15 minutes, and the wells washed twice with TBS. Microscopic examination showed that each microwell was covered by a confluent monolayer of platelets with negligible leukocyte or red cell contamination (<1/1,000 platelets). The fixed platelets could be stored without loss of activity for at least 2 months by filling the wells with a 0.1% (wt/vol) solution of bovine albumin in Dulbecco's modification of Eagle's medium with 20 mmol/L HEPES (pH 7.4) and freezing at −20 °C.

A platelet ELISA[25] inhibition assay was developed using a sheep antimouse F(ab')$_2$ fragment conjugated with horseradish peroxidase and a mouse monoclonal antibody (6D1) directed against an epitope on the GC portion of the platelet GpIb.[8] 6D1 was positive to a titer of 1:160 in a screening assay of inhibition of ristocetin agglutination of fixed platelets in microwells, as previously described.[8] 6D1 was purified from ascites by chromatography on protein A–sepharose, and its specific absorbance at 280 nm was 14. A previously prepared Immulon plate was thawed and washed three times with 10 mmol/L Tris and Tween 20, pH 8.0, (washing buffer). Each microwell was filled with bovine gamma globulin solution (5 mg/100 mL TBS) and incubated for 30 minutes at 37 °C. After three washes with washing buffer, 25 μL of varying concentrations of GC in TBS and 25 μL of 175 ng/mL 6D1 were added to each well. After incubation for 60 minutes at 37 °C and three washes with washing buffer, 50 μL of a 1:250 dilution of the F(ab')$_2$ fragment was added to each well. After incubation for 60 minutes at 37 °C and six washes with washing buffer, 50 μL of a 1:1:18 (vol/vol/vol) mixture of 4% *o*-Phenylenediamine, 0.3% H_2O_2, and 17 mmol/L citric acid in 65 mmol/L phosphate, pH 6.3, was added to each well. After incubation for 30 minutes at 37 °C, the color development was stopped by the addition of 50 μL of 4 5 mmol/L H_2SO_4. The optical density (OD) was read at 492 nm and expressed as a percentage of the OD in assays with 6D1 but without GC (100% binding) The OD of assays with neither 6D1 nor GC was defined as 0% binding. Experiments with ^{125}I-labeled platelets demonstrated that the platelet fixed reproducibly to the microwells and remained adherent throughout the assay (1.51 ± 0.06 × 10^6 platelets fixed per well). 6D1 did not bind in assays without fixed platelets.

Radioiodination of platelet surface proteins. Twenty milliliters of venous blood was drawn into 3 ml of ACD, PRP prepared, the platelet pellet washed three times, and iodination carried out as previously described.[26] The platelets were diluted in TBS, 4 mmol/L EDTA, pH 7.4, to a concentration of $1.5 \times 10^9/\mu$L (approximately 1 mL). A 0.25-mCi sample of ^{125}I in 0.1 N NaOH was added, followed by 5 μL of a 1.1 (vol/vol) mixture of lactoperoxidase and TBS, 4 mmol/EDTA, pH 7.4. Ten microliters of 1 mmol/L H_2O_2 was then added every one minute for ten minutes, after which the platelet pellet was washed as previously described, three or four times (depending on the residual amount of gamma emission measured in the supernatant). Platelets were resuspended in TBS after the last wash.

Serratia protease treatment of platelets. Approximately 4 × 10^8 ^{125}I-labeled platelets in 0.5 mL TBS were incubated at 37 °C with *Serratia marcescens* metalloprotease (final enzyme concentration, 10 μg/mL) for time periods ranging from one to 20 minutes.[27,28] The reaction was stopped by adding 10 mmol/L EDTA[27] and centrifuging the samples at 8,700 g for two minutes. Parallel experiments were performed in which the enzyme solution was replaced by TBS only. For each time period of enzyme and control incubations, a sample of both the platelet pellet and the supernatant was run on SDS gels containing 6% polyacrylamide. An autoradiogram was made of the dried gel with x-ray film at −20 °C.

For the platelet ELISA inhibition assay, 4 × 10^8 washed unlabeled platelets in 0.5 mL TBS were incubated at 37 °C for five minutes with *Serratia* protease (final concentration ~ 10 μg/mL) while another 4 × 10^8 washed unlabeled platelets in 0.5 mL TBS were incubated with TBS only. The enzyme reaction was stopped by adding 10 mmol/L EDTA and centrifuging at 8,700 g for two minutes. The control platelets were treated similarly. The platelets were washed three times with TBS, 4 mmol/L EDTA, pH 7.4, and then diluted in that buffer to a concentration of $10^5/\mu$L. Agglutina-

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tion of *Serratia* protease–treated and control platelets by PRP and ristocetin (1 mg/mL) was assessed by the method of Ramsay and Evatt.[29] The treated and control platelets were then fixed in separate microwells of an Immulon 1 polystyrene plate, as described previously.

Exoglycosidase digestions. Lyophilized GC was resuspended in 0.05 mol/L sodium acetate buffer, pH 6, with 1 mmol/L CaCl. This suspension was sequentially incubated with (1) neuraminidase (0.17 U/μg GC protein) for two hours at 37 °C, (2) β-galactosidase (0.20 U/μg GC protein) overnight at 37 °C, and (3) β-*N*-acetylglucosaminidase (0.02 U/μg GC protein) overnight at room temperature. Before use in the binding assay, all exoglycosidase-treated GC was heated to 50 to 56 °C for ten minutes to inactivate the enzymes Using fetuin and measuring released sialic acid,[30] *o*-nitrophenyl-β-D-galactropyranoside,[31] and *p*-nitrophenyl-2-acetamido-2-deoxy-α-D-glycopyranoside,[31] the activities of neuraminidase, β-galactosidase, and β-*N*-acetyl glucosaminidase, respectively, were standardized and the completeness of heat-induced inactivation verified

Carbohydrate analysis. Unbound sialic acid was measured by the Warren method.[30] Total sialic acid of GC and whole platelets was measured after mild acid hydrolysis with 0.1 N H_2SO_4 for one hour at 80 to 82 °C.[32] Galactose was measured by the method of Finch et al.[33] *N*-acetylglucosamine was measured by a modification of the Morgan and Elson color reaction,[34] as described by Levvy and McAllan.[35] A sample of neuraminidase and β-galactosidase-treated GC was hydrolyzed with 3 N HCl for three hours at 100 °C to release *N*-acetylglucosamine.[5] The pH of the sample was readjusted to 6.0 by dropwise addition of NaOH, and then acetic anhydride dissolved in 1.5% acetone (vol/vol) was added to reacetylate the amino group on the sugar.[35]

Gel Electrophoresis Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber and Osborne[36] as modified by Laemmli.[37] The mobility of the vWF dimer was assessed on 4% polyacrylamide gels and compared to unreduced fibronectin. The mobility of native or exoglycosidase-treated ^{125}I-GC and ^{125}I-labeled platelet proteins was assessed on 7.5% polyacrylamide gels. Autoradiography was carried out on dried gels using Kodak XR film exposed for six to 24 hours at −80 °C with intensifying screens.

Protein determination. GC and vWF protein concentrations were estimated according to the method of Lowry and colleagues.[19]

RESULTS

Inhibition of 6D1 binding to platelets by GC and vWF. As shown in Fig 1, increasing concentrations of purified GC progressively inhibited the binding of monoclonal antibody 6D1 to platelets. The concentration of GC needed to inhibit antibody binding by 50% was 12 nmol/L (Fig 1). In contrast, the binding of 6D1 to platelets was not inhibited when 1 μmol/L fetuin, another heavily glycosylated protein, was substituted for GC (data not shown). In addition, GC did not inhibit the binding to platelets of KB 2/1, a monoclonal antibody directed at another protein on the platelet membrane (Fig 1).

Pretreatment of platelets for five minutes with the protease purified from *Serratia marcescens,* which has been reported to selectively remove GpIb from the platelet surface,[27,28] decreased 6D1 antibody binding by 75% and abolished the platelets' ability to be agglutinated by ristocetin in the presence of normal plasma. In contrast, the protease treatment had no effect on the binding of antibody KB 2/1. SDS-PAGE analysis of nonreduced samples of the protease-treated platelets showed complete loss of the GpIb band with the appearance in the supernatant of a new band migrating with an <M$_r$> similar to, or identical with, that of GC.

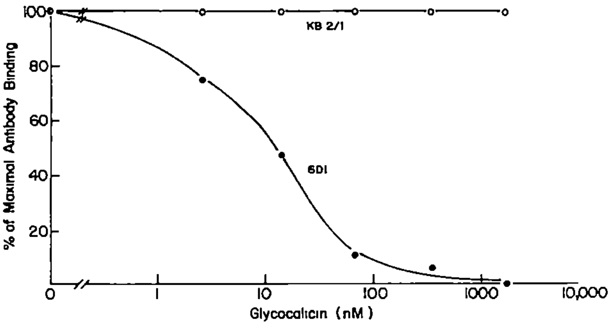

Fig 1. Effect of GC on the binding of two monoclonal antibodies to platelets. Percent binding of antibody to fixed platelets was measured by an ELISA. 6D1 (88 ng/mL) (●) was a monoclonal antibody directed at platelet GpIb. KB 2/1 (○) was a monoclonal antiplatelet membrane antibody that is not directed at GpIb. Each point represents the mean of four experiments (SEM ± 7% to 8%).

The inhibition of 6D1 binding to whole platelets by vWF was then assessed. Binding of 88 ng/mL 6D1 to formalin-treated platelets was not inhibited by simultaneous incubation with up to 43 μg/mL vWF alone. However, the addition of 1.5 mg/mL ristocetin along with 43 μg/mL vWF inhibited 6D1 binding by up to 67%. These studies suggest that the epitope on platelet GpIb and purified GC that is recognized by 6D1 is either located near the binding site for vWF or altered by vWF binding at a site remote from it.

Inhibition of vWF binding to platelets by GC. GC inhibited the ristocetin-dependent binding of the ^{125}I-labeled vWF dimer to the platelet surface (Fig 2). The ^{125}I-vWF dimer bound to platelets in the presence of ristocetin in a saturable and reversible manner and could be inhibited by GC. The IC_{50} for GC using vWF as radioligand was 10 nmol/L (Fig 2). In contrast, vWF binding to platelets was not inhibited when 1 μmol/L fetuin was substituted for GC.

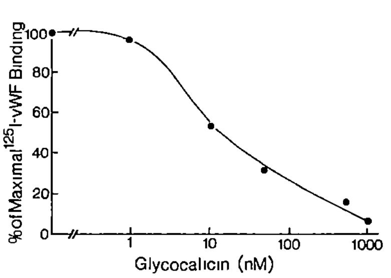

Fig 2. Effect of GC on ristocetin-dependent binding of ^{125}I-vWF to platelets. Binding assay was performed as described in Methods using fresh washed platelets at a concentration of 2 × 10^4/μL, ^{125}I-labeled vWF 0.48 μg/mL, 1 mg/mL ristocetin, and the indicated concentrations of purified GC. Specific binding of vWF was determined by subtracting the results of parallel assays without ristocetin as a measure of nonspecific binding (typically comprising 20% of total binding). Each point represents the mean of three experiments (SEM ± 8% to 9%).

Table 1. Release of Monosaccharides by Combinations of Exoglycosidases

Exoglycosidase Added	Monosaccharide Released (N Moles)*		
	NANA	GAL	*N*-Ac-Glu
Neuraminidase	40.5 ± 5.0	1.2 ± 0.6	3.2 ± 0.4
Neuraminidase and *β*-galactosidase	—	39.5 ± 4.0	3.5 ± 0.5
Neuraminidase, *β*-galactosidase, and *β*-*N*-acetyl glucosaminidase	—	—	10.8 ± 2.0
Acid hydrolysis	44.3 ± 6.0	40.5 ± 4.0	19.5 ± 3.5

Abbreviations: NANA, *N*-acetyl neuraminic acid; GAL, galactose; *N*-Ac-Glu, *N*-acetyl glucosamine.

*Mean ± SEM for four to five separate experiments each done in duplicate.

Effect of exoglycosidase treatment on monosaccharide content and GC-antibody interactions. The ability of native and exoglycosidase-treated GC to inhibit 6D1 antibody binding to platelets was then assessed. Table 1 shows the quantity of monosaccharide released from GC by enzyme incubation. Neuraminidase treatment of GC released 40.5 of a total of 44.3 nmol of sialic acid. Combined neuraminidase and *β*-galactosidase treatment cleaved 39.5 of 40.5 nmol of galactose, and treatment with all three enzymes—neuraminidase, *β*-galactosidase, and *β*-*N*-acetyl glucosaminidase—released 10.8 of a total of 19.5 nmol of *N*-acetyl glucosamine from GC. As shown in the inset to Fig 3, exoglycosidase digestion of GC did not cause significant proteolysis of GC. Several minor bands with $<M_r>$ less then native GC can be seen on the autoradiograms of ^{125}I-GC prior to enzyme treatment. However, the intensity of these bands did not increase after exposure to the exoglycosidases. They represent less than 1% of the total radioactivity. It is interesting, however, that treatment with neuraminidase (lane 2) reduced the mobility of ^{125}I-GC slightly, whereas treatment with additional exoglycosidases (lanes 4 and 5) did not further reduce mobility.

The ability of GC to inhibit 6D1 binding to platelets was not affected by sequential enzyme digestion. Following treatment of GC with neuraminidase alone, neuraminidase and *β*-galactosidase, or the combination of neuraminidase, *β*-galactosidase and *β*-*N*-acetyl glucosaminidase, GC still inhibited 6D1 antibody binding to platelets (Fig 3). In addition, pretreatment of intact platelets with neuraminidase, which removed 49% of the total platelet sialic acid, did not affect subsequent binding of 6D1 to the platelet. Finally, each of the four monosaccharides that make up the carbohydrate sequences on GC[6,16] was substituted for GC in the platelet ELISA assay. None of the four monosaccharides tested—*N*-acetyl neuraminic acid, galactose, *N*-acetyl glucosamine, *N*-acetyl galactosamine—when added over a concentration range of 100 nmol/L to 1 mmol/L, inhibited binding of antibody 6D1.

Effect of exoglycosidase treatment on GC-vWF interactions. In contrast to 6D1 binding, exoglycosidase digestion of GC did modify the interaction of GC and vWF. The ability of GC to inhibit vWF binding to platelets in the presence of ristocetin was not altered by prior treatment of GC with neuraminidase alone nor with the combination of neuraminidase and *β*-galactosidase, as assessed in the ^{125}I-vWF/platelet binding assay (Fig 4). However, sequential treatment of GC with all three enzymes—neuraminidase, *β*-galactosidase, and *β*-*N*-acetyl glucosaminidase—caused a 40-fold increase in the IC_{50} (Fig 4). To be certain that the exoglycosidases present in some of the GC samples did not hydrolyze monosaccharides from the platelet or from vWF and thereby inhibit vWF binding to platelets, we heated enzyme-treated and control GC samples to 50 to 56 °C for

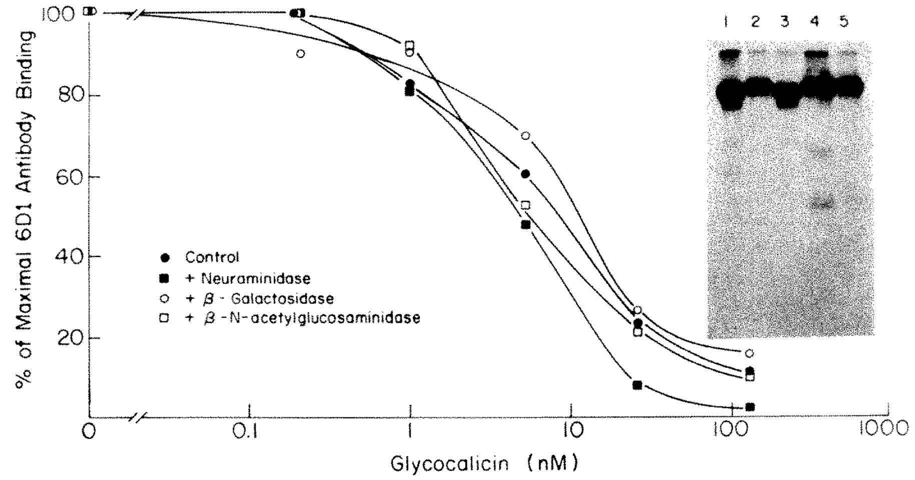

Fig 3. Effect of exoglycosidase-treated GC on the binding of monoclonal antibody 6D1 to platelets. Percent binding of 6D1 (88 ng/mL) to fixed platelets was measured by ELISA. Key: ●, control GC; ■, neuraminidase-treated GC; ○, neuraminidase- and *β*-galactosidase-treated GC; □, neuraminidase-, *β*-galactosidase-, and *β*-*N*-acetyl glucosaminidase-treated GC. The inset depicts an autoradiogram of control and exoglycosidase-treated GC after 5% SDS-PAGE. Lanes 1 and 3, no treatment; lane 2, neuraminidase; lane 4, neuraminidase and *β*-galactosidase; lane 5, neuraminidase, *β*-galactosidase, and *β*-*N*-acetyl glucosaminidase. Each point represents the mean of three experiments (SEM ± 6% to 7%).

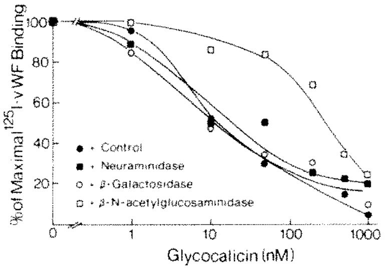

Fig 4. Effect of exoglycosidase-treated GC on ristocetin-dependent binding of ^{125}i-vWF to platelets. Binding assay performed as described in Methods. Reagents: fresh washed platelets, 2 × $10^4/\mu L$; unlabeled and ^{125}I-labeled vWF, 0.48 μg/mL; ristocetin, 1 mg/mL. Key: ●, control GC; ■, neuraminidase-treated GC; ○, neuraminidase- and β-galactosidase-treated GC; □, Neuraminidase-, β-galactosidase-, and β-*N*-acetyl glucosaminidase-treated GC. Each point represents the mean of two experiments.

ten minutes to inactivate the enzymes, which completely inhibited activity of these glycosidases against standard substrates.

DISCUSSION

In this paper, we present evidence that GC, a soluble glycoprotein derived from platelet membrane GpIb, contains a binding site for vWF. First, the binding to platelets of a monoclonal antibody directed at an epitope on GpIb was inhibited (1) both by the ristocetin-dependent binding of vWF, and (2) the addition of purified GC. Second, GC completely inhibited ristocetin-dependent binding of ^{125}I-labeled vWF to the platelet surface.

Other investigators have provided some qualitative evidence that GC contains a platelet vWF receptor of GpIb.[2,7] Platelets from patients with the Bernard-Soulier syndrome completely lack GpIb[38] and do not bind vWF in the presence of ristocetin.[39] Ali-Briggs et al[40] demonstrated that a heterologous polyclonal antibody to GpIb inhibited ristocetin-induced platelet agglutination. Coller et al subsequently developed a monoclonal antibody (6D1) against GpIb that inhibited bovine vWF-induced platelet agglutination.[8] The addition of purified GC reversed this inhibition, suggesting that the antibody was directed at an epitope present on GC as well as GpIb. Okumura and Jamieson[9] previously reported that 1 μmol/L GC inhibited ristocetin-induced agglutination of human platelets by vWF. The data presented here provide further evidence that the binding site for vWF is located on GC and indicate in a quantitative fashion the magnitude of the affinity of GC for this ligand.

In previous studies, Coller[8] determined that monoclonal antibody 6D1 bound to whole platelets with a K_d of approximately 15 nmol/L. Our finding of an IC_{50} of 12 nmol/L for GC inhibition of 6D1 binding to whole platelets suggests that 6D1 antibody binds with equal avidity to GC and GpIb and that the conformation of this epitope is not altered during the cleavage and purification of GC. In contrast, GC inhibited vWF binding to platelets with an IC_{50} of 10 nmol/L, whereas the K_d for the binding to platelets of vWF was 30 nmol/L. This threefold difference may be due to steric effects present on the platelet surface that are mitigated by cleaving GC from that surface.

To determine whether carbohydrate sequences on GC were necessary for wWF binding, GC was incubated with a series of exoglycosidases. Since the sequence of the major oligosaccharide chain of GC has been determined to be *N*-acetyl neuraminic acid, galactose, *N*-acetyl glucosamine, and *N*-acetyl galactosamine,[16] we treated GC sequentially with neuraminidase, β-galactosidase and β-*N*-acetyl glucosaminidase. The results suggest that sialic acid and galactose are not part of the binding site for vWF on the GC portion of GpIb. Partial removal of *N*-acetyl glucosamine residues on GC selectively reduced the binding of vWF, but not 6D1 antibody. Thus, these residues may not be an essential part of the epitope recognized by the 6D1 antibody, but they are required for vWF binding to GC either because they are part of the vWF binding site on GC or because their removal induces a conformational change in the GC molecule. Although the simplest conclusion from these data is that the binding sites for vWF and 6D1 are different (as further supported by the lack of complete inhibition of 6D1 binding to platelets by vWF presented in Results), the possibility remains that the glycosidase treatment results in alterations at an accessory site(s) involved in vWF, but not 6D1, binding and that the major binding sites are, in fact, identical.

Treatment of whole platelets with neuraminidase released 49% of the total platelet sialic acid, a similar percentage to that reported by others.[41-43] The fact that removal of surface sialic acid did not diminish binding of 6D1 antibody to platelets is in accordance with the findings of others as well.[43-45] Neuraminidase treatment of platelets did not diminish their agglutination by ristocetin,[43] their agglutination by bovine factor VIII,[44] or their ability to bind AN51, a monoclonal antibody to GpIb.[45] These experiments are all consistent with the hypothesis that sialic acid is not a part of the platelet vWF receptor on GC/GpIb.

There is evidence that carbohydrate residues on other cell surface proteins may contribute to their biologic activity, such as glycophorin[46] and blood group antigens, ABH and Ii[47] on erythrocytes, the insulin receptor on preadipocytes,[48] and glycoprotein antigens on viruses.[49] Although these whole-cell studies are of great interest, the relationship between carbohydrate modification and receptor activity can only be inferred since many cell surface structures may be modified. This criticism does not apply to the data presented here since purified GC was used as a substrate for exoglycosidases.

Our studies provide an example of carbohydrate-mediated receptor binding that may be unique in several ways. First, GC is a soluble receptor fragment that retains biologic activity after purification and can be studied without lipid cofactors or detergent micelles. Second, it was possible to modify carbohydrate sequences on the purified receptor fragment and then quantify and correlate these modifications with changes in biologic activity. Third, we were able to compare the effect of these carbohydrate modifications on the epitope recognized by a monoclonal antibody with their effect on ligand (ie, vWF) binding. Future studies of this system will be directed towards localizing the vWF binding site to a specific region of the GC molecule and comparing the binding characteristics of soluble and lipid-bound forms of this receptor.

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A Diagnostic Test for Heparin-Induced Thrombocytopenia

By David Sheridan, Cedric Carter, and John G. Kelton

Heparin-induced thrombocytopenia can be a serious and difficult-to-diagnose complication of heparin therapy. Serum from patients with heparin-induced thrombocytopenia can cause heparin-dependent platelet aggregation, but the low sensitivity and specificity of this test limit its clinical usefulness. In this report we describe an assay for heparin-induced thrombocytopenia that is both sensitive and specific. The improvement in the assay was accomplished by measuring platelet release instead of aggregation and by measuring platelet release at two heparin concentrations. The rationale for the use of two heparin concentrations was that sera from patients with heparin-induced thrombocytopenia caused release at therapeutic but not at high concentrations of heparin. Twenty-eight sera samples from patients suspected of having heparin-induced thrombocytopenia and 573 controls were coded and tested in the assay. The patients with possible heparin-induced thrombocytopenia were ranked according to the likelihood of having this disorder by using prospectively defined criteria. The test had a high specificity (99%); only one of 573 controls showed a positive result. The test was also very sensitive, and the likelihood of a positive test result was directly correlated with the clinical likelihood of the patient having heparin-induced thrombocytopenia. Six of six patients with definitive heparin-induced thrombocytopenia had positive test results, whereas zero of four patients in whom the diagnosis was unlikely had positive test results. The two-point test for heparin-induced thrombocytopenia represents a sensitive and specific test for this disorder. This test may be useful not only in confirming the diagnosis of this disorder but also may provide information about its pathogenesis.

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HEPARIN IS AN EFFECTIVE anticoagulant for the prevention and the treatment of venous thrombosis. Thrombocytopenia develops in approximately 5% to 10% of patients treated with heparin.[1-4] Usually this complication has little clinical impact, but thrombocytopenia in association with arterial thromboses develops in a small proportion of patients treated with heparin.[2] This adverse reaction, unlike isolated heparin-induced thrombocytopenia, results in considerable morbidity, including the occlusion of the arteries supplying the heart, brain, and extremities. In these circumstances it is necessary to discontinue heparin therapy as quickly as possible.[2]

Many of the patients receiving heparin have comorbid conditions that predispose them to thrombocytopenia resulting from other causes, and it can be difficult to be certain of the diagnosis of heparin-induced thrombocytopenia. Yet it is important to be as certain as possible of the diagnosis of heparin-induced thrombocytopenia since discontinuation of the heparin often requires the institution of other antithrombotic therapies such as defibrination or venous ligation that by themselves can result in morbidity. Patients with heparin-induced thrombocytopenia have elevated levels of platelet-associated IgG, but the low specificity of this finding makes a positive result of limited value.[5,6] Previous investigators have demonstrated a heparin-dependent platelet-aggregating factor in sera of patients with heparin-induced thrombocytopenia,[7] and it was suggested that this test could be used to diagnose heparin-induced thrombocytopenia. However, in a prospective study evaluating the usefulness of testing for heparin-dependent platelet aggregation, we found that this response does not occur in every patient.[6] Furthermore, the result of the test is positive in some patients without heparin-induced thrombocytopenia. Problems with both sensitivity and specificity seriously compromise the diagnostic usefulness of any test.

During the performance of our previous study, we made an unexpected observation: sera from patients with heparin-induced thrombocytopenia would initiate platelet aggregation at therapeutic concentrations of heparin, but this response did not occur at high concentrations of heparin. We also observed that ^{14}C-serotonin platelet release was a more sensitive test of platelet activation than was aggregation. These two observations were exploited to develop a sensitive and specific test for confirming the diagnosis of heparin-induced thrombocytopenia.

MATERIALS AND METHODS

Materials. The following materials were used. porcine heparin (Hepalean, Harris Laboratories, Toronto); 5-hydroxy-^{14}C-tryptamine creatinine sulphate (Amersham Corp, Oakville, Canada); calcium- and albumin-free Tyrode's solution containing apyrase, pH 6.2; albumin-free Tyrode's solution, pH 7.4; aqueous counting scintillant (ACS; Amersham Corp)

Sample collection. Serum and plasma samples were obtained from patients in the Hamilton area. The samples from the thrombocytopenic patients receiving heparin were collected within two days of development of the thrombocytopenia. These samples were obtained from subsamples that were collected as part of routine patient management. All samples were heat inactivated for 60 minutes at 56 °C Plasma samples were centrifuged for five minutes at 8,000 g after heat inactivation to remove the fibrin gel, and the supernatant serum was removed. All samples were stored at −70 °C until use

Platelet preparation. Whole blood from healthy individuals was collected into ACD (acid-citrate-dextrose, 1:6, vol:vol) The platelet-rich plasma (PRP) was obtained by centrifugation for 15 minutes at 250 g. The PRP was incubated with ^{14}C-serotonin for 45 minutes at 37 °C (50 μCi ^{14}C-serotonin per mL of PRP) Approximately 50% of

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the ^{14}C-serotonin bound to the washed platelets, giving a final specific activity of 1.84×10^{-4} μCi of serotonin per 10^6 platelets. The platelets were washed once in calcium- and albumin-free Tyrode's solution and were resuspended in albumin-free Tyrode's solution. The final platelet count was adjusted to 300,000/μL.

Serotonin release assay. Twenty microliters of test serum was mixed with 5 μL of one of two heparin concentrations (0.1 and 100 U/mL, final) and 75 μL of ^{14}C-serotonin–labeled platelets. The platelet mixture was incubated in microtiter wells that contained a magnetic stir bar. Following a 60-minute incubation at 22 °C on a magnetic stir plate set at a slow speed, 100 μL of 0.5% EDTA in saline was added to each well to terminate the release reaction. The platelets were centrifuged for five minutes at 1,500 g, and 50 μL of the supernatant fluid was removed. This aliquot was added to 10 mL of ACS, and the samples were counted in a liquid scintillation counter. The percent release was calculated as follows:

$$\text{percent release} = \frac{\text{release of test sample} - \text{background}}{\text{total radioactivity} - \text{background}} \times 100$$

The background was defined as the supernatant fluid radioactivity from platelets that were handled identically to the test platelets except that buffer was substituted for serum. The total platelet radioactivity was the radioactivity of the test platelet sample. On the basis of preliminary studies, a test result was defined as positive if both of the following criteria were fulfilled greater than 20% release at 0.1 U/mL heparin and less than 20% release at 100 U/mL heparin.

Preliminary studies. Sera from three patients with definite (see subsequent criteria) heparin-induced thrombocytopenia and three normal controls were tested for heparin-dependent ^{14}C-serotonin release. The concentrations of heparin used for these preliminary experiments ranged from 0 to 1,000 U/mL. None of the normal control samples tested induced serotonin release at any of these concentrations, whereas the heparin-induced thrombocytopenia patient samples induced maximum release at therapeutic heparin concentrations (Fig 1).

Patients with possible heparin-induced thrombocytopenia. Sera or plasma from 28 patients with possible heparin-induced thrombocytopenia were available. To categorize these patients, we developed clinical criteria to estimate the likelihood of a particular patient having heparin-induced thrombocytopenia (Table 1). Each patient's hospital record was reviewed by a single individual and the patient classified according to the criteria of Table 1. The patients were then grouped according to their classification, and the results of the assay were compared among groups.

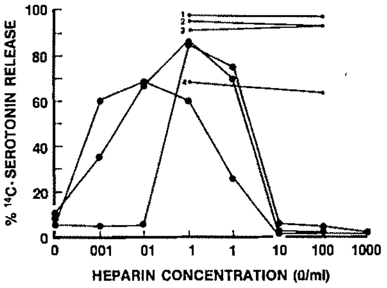

Fig 1. The relationship between the percent release of ^{14}C-serotonin from platelets obtained from healthy individuals (ordinate) and the final concentration of heparin (abscissa). Each series of points is the result obtained with serum from a different patient with definite heparin-induced thrombocytopenia. The lines numbered 1 to 4 represent four different sera samples not from patients with heparin-induced thrombocytopenia. Those samples induced release at 0.1 and 100 U/mL of heparin. Numbers 1 and 2 are sera from two patients with ITP. Number 3 is the serum from a hospitalized control, and number 4 is heat-aggregated IgG.

Table 1. Clinical Criteria for Estimating the Likelihood That a Patient Has Heparin-Induced Thrombocytopenia

- Definite heparin-induced thrombocytopenia
  - Thrombocytopenia develops in patient while receiving heparin, and other causes of thrombocytopenia are excluded. Thrombocytopenia recurs when the patient is rechallenged with heparin, *or*
  - Thrombocytopenia plus acute arterial thrombosis, with other causes of arterial thrombosis excluded.
- Probable heparin-induced thrombocytopenia
  - Thrombocytopenia develops in patient while receiving heparin; *and*
  - Other causes of thrombocytopenia excluded, ie, blood culture is negative; other drugs excluded.
- Possible heparin-induced thrombocytopenia
  - Patient fulfills second criteria, but the thrombocytopenia resolves during continued heparin therapy; *or*
  - Patient fulfills second criteria, but thrombocytopenia does not recur with subsequent administration of heparin (negative rechallenge).
- Heparin-induced thrombocytopenia is unlikely
  - Thrombocytopenia develops in patient while receiving heparin, but another cause for the thrombocytopenia is found; *or*
  - The thrombocytopenia does not resolve after the heparin is discontinued.

Controls The controls consisted of 15 healthy laboratory personnel, 408 unselected hospitalized patients not receiving heparin, 50 hospitalized nonthrombocytopenic patients receiving heparin, and 100 unselected thrombocytopenic patients not receiving heparin. After the test was completed, a positive result was noted in one of the heparin controls, and a review of this individual's chart demonstrated that he had become thrombocytopenic on the day of testing.

Heat-aggregated human IgG served as a positive control for platelet release. Human IgG (Cohn II) was heat aggregated at 63 °C for 20 minutes. This was then incubated with the platelets in place of the serum, and the percent release measured in the usual way.

The patient and control samples were tested at the two heparin concentrations by a single individual who was blinded as to their origin The result was considered positive for heparin-induced thrombocytopenia only if release (greater than 20%) occurred at a heparin concentration of 0.1 U/mL and did not occur at 100 U/mL of heparin.

RESULTS

When attempting to diagnose a relatively uncommon disease, test specificity is of primary importance. The test described in this report was highly specific for heparin-induced thrombocytopenia (Table 1). Only one of 573 control samples showed positive results. The controls included healthy controls (n = 15), nonthrombocytopenic heparin controls (n = 50), hospitalized patients not receiving heparin (n = 408), and patients with thrombocytopenia not resulting from heparin (n = 100). There was only one false-positive result (release at therapeutic concentrations of heparin, 0.1 U/mL, but not at high concentrations of heparin, 100 U/mL). This occurred in a 12-year-old girl with idiopathic thrombocytopenic purpura (ITP) who had never received heparin. One heparin control showed positive test results, but this patient was found to be thrombocytopenic on the day of testing.

The importance of testing for release at high- and low-heparin concentrations is illustrated by the observation that had we defined a positive result only as requiring ^{14}C-serotonin release at a therapeutic heparin concentration (0.1 U/mL), then 21 of the controls would have been misclassified as having heparin-induced thrombocytopenia (Table 2). Many of these patients had ITP, and their sera induced ^{14}C-serotonin release even without the addition of any heparin. These patients clearly represented a group separate from the heparin-induced thrombocytopenia patients since only one of these patients demonstrated inhibition of release at the 100-U/mL concentration of heparin.

The test was sensitive for the detection of heparin-induced thrombocytopenia, and there was a strong relationship between the likelihood of a patient having heparin-induced thrombocytopenia and a positive result in the assay. Six of six patients with definite heparin-induced thrombocytopenia (Table 3) had a positive result, whereas none of the four patients in the fourth group (heparin-induced thrombocytopenia unlikely) and only two of ten in the third group were positive.

The test was positive regardless of whether the patient was receiving heparin at the time of testing or not. Three patients who were classified as having definite heparin-induced thrombocytopenia were receiving heparin at the time the test was performed, and positive results occurred in all. The sera samples from these patients induced platelet release without the addition of heparin, although the addition of 1.0 U/mL of heparin to the test serum did not inhibit the release reaction, as could be predicted from Fig 1.

Platelet release induced by immune complexes (heat-aggregated IgG) was not affected by either concentration of heparin and ranged from 60% to 90% (Fig 1).

The level of platelet-associated IgG was measured at the time of thrombocytopenia in seven of the patients with suspected heparin-induced thrombocytopenia and was elevated in six.

DISCUSSION

Heparin-induced thrombocytopenia is a complication of heparin therapy that can result in morbidity and occasionally death.[1-4] The diagnosis remains one of exclusion because there is no highly sensitive or specific diagnostic test for this disorder.[5,6] Therefore, clinical decisions must be based on diagnostic probabilities. The implications of continuing or discontinuing heparin therapy are considerable, and it would be useful to have a test that could confirm or exclude heparin-induced thrombocytopenia. The in vitro test described in this report represents such a test and is both sensitive and specific for confirming the diagnosis of heparin-induced thrombocytopenia.

Table 2. The Results of the In Vitro Test for Heparin-Induced Thrombocytopenia for 28 Thrombocytopenic Patients Who Were Receiving Heparin

Classification	Number	Number With Positive In Vitro Test Results
Definitive heparin-induced thrombocytopenia	6	6
Probable heparin-induced thrombocytopenia	8	5
Possible heparin-induced thrombocytopenia	10	2
Heparin-induced thrombocytopenia unlikely	4	0

Table 3. Results of Measuring Heparin-Dependent Serotonin Release in the Various Control Populations

Pattern of Serotonin Release	Heparin Concentration (Final)	Number of Various Control Subjects			
		Healthy Controls	Hospital Controls	ITP Patients	Heparin Controls (Nonthrombocytopenic)
<20%	0.1 U	15	395	92	40
<20%	100 U				
>20%	0.1 U	0	13	7	1
>20%	100 U				
>20%*	0.1 U	0	0	1	1†
<20%*	100 U				

*Includes those controls with a positive test (as defined in the text) for heparin-induced thrombocytopenia

†This patient was discovered to be thrombocytopenic at the time of testing.

The technique is based upon two observations: (1) heparin-dependent platelet ^{14}C-serotonin release occurs at therapeutic concentrations of heparin, and (2) very high concentrations of heparin inhibit the release reaction. Others have reported, and we have confirmed, that sera from some patients with heparin-induced thrombocytopenia can induce platelet aggregation in the presence of heparin.[6,7] But, this in vitro test is neither sensitive nor specific.[6] The method described in this report represents an improvement in both sensitivity and specificity. To increase the sensitivity we measured ^{14}C-serotonin release instead of platelet aggregation since immunologic stimuli can induce release without aggregation.[8] The problem with the use of a single concentration of heparin and measuring platelet release as a test for heparin-induced thrombocytopenia is its low specificity. Sera from patients with other thrombocytopenic disorders also can induce platelet release. This difficulty was overcome by assessing the release reaction at two heparin concentrations. Preliminary studies showed that heparin-dependent platelet release was unimodal (Fig 1). Sera from patients with heparin-induced thrombocytopenia caused platelet release at therapeutic concentrations of heparin, but high concentrations of heparin inhibited the reaction. Subsequent studies, not described in this report, have demonstrated that the plasma factor that causes heparin-dependent platelet release is IgG. The unimodal pattern of release may provide insight into the spatial orientation of the binding of the antibody-heparin complex to the platelet. For example, a similar unimodal reaction was observed with malaria-induced thrombocytopenia in which the binding of IgG was directed at platelet-bound antigens.[9] However, unimodal binding also characterizes certain immune complex interactions. Consequently, the characterization of the precise type of binding of IgG-heparin to the platelet surface probably will require the use of immunoglobulin digests.

The inclusion of the high-heparin concentration was the primary factor explaining the increased specificity. Using this test modification, none of the patients that were classified clinically as having definite heparin-induced thrombocytopenia had negative results; however, 20 of 21 control patients whose sera induced release with low concentrations of heparin continued to cause release at high-heparin concentrations. The high specificity of the test for heparin-induced thrombocytopenia was confirmed by testing many other sera samples from patients without this disorder. However, it is more difficult to evaluate test sensitivity because there is not a diagnostic "gold standard" for heparin-induced thrombocytopenia For this reason, we related the in vitro results to a clinical estimate of the likelihood that the patient had heparin-induced thrombocytopenia. We found that those patients with the highest clinical probability of having heparin-induced thrombocytopenia also had the highest positivity rate in the assay. And as the clinical probability of a patient having heparin-induced thrombocytopenia decreased, so did the likelihood of a positive test. It is theoretically possible that other heparin concentrations could be used in the diagnostic test. For example, Fig 1 indicates that a no-heparin-added test in addition to the 0.1-unit test could be as sensitive as the 0.1-unit and the 100-unit test in detecting heparin-induced thrombocytopenia. However, this approach is not always clinically practical since it requires the cessation of heparin therapy to perform the test.

In summary, this study indicates that the measurement of heparin-dependent platelet release at two heparin concentrations can be used as a specific diagnostic test for heparin-induced thrombocytopenia. It is possible that this test also can be used to provide insight into the pathophysiology of heparin-induced thrombocytopenia.

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Biosynthetic (Recombinant) Human Granulocyte-Macrophage Colony-Stimulating Factor: Effect on Normal Bone Marrow and Leukemia Cell Lines

By Masao Tomonaga, David W. Golde, and Judith C. Gasson

To examine the biologic properties of the molecule encoded by the human gene for granulocyte-macrophage colony-stimulating factor (GM-CSF), we expressed the cloned complementary DNA (cDNA) in transfected monkey COS cells and purified the resultant protein. Purified biosynthetic human GM-CSF was added to cultures of normal hematopoietic progenitor cells in semisolid media, and the resulting colonies were characterized cytochemically. Nonadherent light-density bone marrow cells from healthy adult volunteers were maximally stimulated with GM-CSF (approximately 250 pmol/L, and four types of colonies were consistently identified by aspirating the individual colonies and staining with a triple stain for specific and nonspecific esterases and eosinophilic granules. Pure neutrophilic granulocyte (G), mixed granulocyte-macrophage (GM), pure macrophage (M), and pure eosinophil (EO) colonies were observed, the mean incidences on day 8 being 70%, 20%, 5%, and 5%, and on day 14, 7.5%, 16.6%, 50.9%, and 25.0%, respectively. In all types of colonies, complete maturation to segmented forms or typical macrophages was detected. GM-CSF did not enhance the growth of BFU-E from normal peripheral blood buffy coat cells in the simultaneous presence of erythropoietin alone or erythropoietin with purified erythroid-potentiating activity. GM-CSF stimulated HL-60 and KG-1 colony formation twofold and fivefold, respectively; consistent differentiation induction towards monocytic and eosinophilic lineages was observed in HL-60 but not in KG-1. These in vitro findings indicate that GM-CSF is a multilineage stimulator for progenitor cells of G, GM, M, and EO colonies.

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COLONY-STIMULATING FACTORS (CSFs) are essential for hematopoietic progenitor cells to proliferate and differentiate in vitro,[1-4] and they likely play an important role in vivo.[5-7] Accurate assessment of the interaction of CSFs with their target cells requires the availability of purified hormone. In this context, murine CSFs have been well characterized, and four distinct molecules are now known to stimulate myeloid progenitors: macrophage CSF (M-CSF) stimulates the growth of macrophage progenitors,[8] granulocyte-macrophage CSF (GM-CSF) acts on both neutrophilic granulocyte and macrophage progenitors,[9] granulocyte CSF (G-CSF) primarily stimulates granulocyte progenitors,[10] and multi-CSF (interleukin 3) has effects on multiple progenitors of erythroid, eosinophilic (EO), megakaryocytic, and mast cell lineages as well as granulocyte and macrophage lineages.[11] Recently, molecular clones encoding murine IL-3[12,13] and GM-CSF[14] have been isolated and characterized.

Human CSFs have been less well characterized. Nicola et al separated two types of CSF from human placental-conditioned medium on the basis of their hydrophobicity[15]: the first activity (CSFα) contains GM-CSF, EO-CSF, and possibly erythroid-CSF[3] activities; and the second one (CSFβ) is very similar to murine G-CSF. Comparable results were reported with conditioned medium from the GCT cell line.[16] We recently purified a 22,000–molecular weight (mol wt) GM-CSF from medium conditioned by the Mo-T-lymphoblast cell line.[17] Complementary DNA clones encoding this human T cell–derived GM-CSF have been isolated, and there is about 60% homology with the nucleotide sequence of the murine GM-CSF cDNA.[18] Purified natural and biosynthetic human GM-CSF have activities on precursor cells, as well as mature neutrophils.[17,19] In this study we define the biologic activity of the purified biosynthetic GM-CSF on human hematopoietic progenitors using in vitro clonal culture of normal blood and bone marrow cells and the human myeloid leukemia cell lines HL-60 and KG-1.

MATERIALS AND METHODS

Cell Preparations

Bone marrow was obtained with informed consent from healthy volunteers by aspiration from iliac crest. Light-density cells were separated by Ficoll-Hypaque density gradient centrifugation. Five million light-density cells suspended in 5 mL of Iscove's modified Eagle's medium (MEM) (Irvine Scientific, Santa Ana, Calif) containing 20% fetal bovine serum (FBS; Hyclone, Logan, Utah) were incubated in a Falcon T-flask (Becton Dickinson, Oxnard, Calif) for two hours at 37 °C in 5% CO_2 in air to deplete adherent cells. Nonadherent light-density cells were gently harvested, washed twice in complete medium, and used for the CFU-GM assay.

For the BFU-E assay, peripheral blood buffy coat cells were treated with Tris-HCL (pH 7.56)-buffered 0.89% ammonium chloride for 9.5 minutes at 37 °C to lyse the contaminating red blood cells The nucleated cells were washed twice with Iscove's MEM containing 10% FBS.

Two human myeloid cell lines, HL-60[20] and KG-1,[21] were used for differentiation-inducing experiments by GM-CSF. Both cell lines have been maintained in Iscove's MEM containing 10% FBS. Cells harvested during exponential growth were used for the experiments

Hematopoietic Stimulators

Purified biosynthetic GM-CSF was obtained from serum-free medium conditioned by COS monkey kidney cells transfected with the GM-CSF cDNA clone in the expression vector p91023 (B).[17,18] The GM-CSF was purified from the conditioned medium by a

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modification of the previous method[17] using only gel filtration and reverse-phase high-performance liquid chromatography. The lectin affinity column could be eliminated because of the greater abundance of biosynthetic GM-CSF in the conditioned medium. The purified GM-CSF appeared as a single protein band of 22,000 mol wt on sodium dodecylsulfate polyacrylamide gel electrophoresis, the same molecular weight as the purified natural GM-CSF.[17] The concentration of GM-CSF in a reference preparation was estimated by amino acid analysis; the specific activity of this purified material on normal bone marrow cells is 2×10^8 U/mg. Half maximal activity on bone marrow cells is seen at 10^{-11} mol/L.

Erythroid-potentiating activity (EPA) used in the BFU-E assay was purified from Mo-T cell-conditioned medium as previously reported.[22] Human urinary erythropoietin (EPO) was provided by the Heart, Lung and Blood Institute (Bethesda, Md). The EPO preparation (CAT-1) had a specific activity of 1,140 U/mg protein. The hematopoietic stimulators were serially diluted to various concentrations with Iscove's MEM just before addition to the cultures.

Clonal Culture of Hematopoietic Progenitors in Methylcellulose

Light-density nonadherent bone marrow cells (final concentration, 2.5 to 5×10^4/mL) were plated in Iscove's MEM containing 0.88% methylcellulose, 20% FBS, and GM-CSF at various dilutions in Iscove's MEM. One-half milliliter of semisolid medium was plated in 2.0-cm^2 Falcon well for colony analysis, and 0.1 ml was plated in a Falcon microtiter well for the GM-CSF dose-response studies.

BFU-E assays were performed according to the micromethod previously reported.[22,23] Peripheral blood buffy coat cells (final concentration, 3.3×10^5/mL) were plated in Iscove's MEM containing 0.8% methylcellulose, 20% FBS, 10^{-4} mol/L alpha-thioglycerol, 0.33 U/mL EPO, and 10% volumes of diluted EPA and/or GM-CSF.

CFU-GM colonies were enumerated on day 8 in all cultures and also on day 14 in some, counting cell aggregates containing 20 cells or more as CFU-GM. Erythroid bursts were enumerated on day 14. Well-hemoglobinized colonies containing 100 or more cells were regarded as BFU-E. They were confirmed as having hemoglobin with diaminobenzidine according to Ogawa et al.[24]

Cytochemical Staining of Individual CFU-GM Colonies

After enumerating the number of CFU-GM, individual colonies were aspirated using a finely drawn-out microcapillary tube, carefully avoiding contamination with an adjacent colony or cluster. The aspirated colony was then transferred into a 10-µL droplet of Iscove's MEM placed on a poly-L-lysine-coated glass slide.[25] After complete attachment of colony cells onto the glass surface, medium was removed with absorbent paper and the slides dried rapidly with air. A slide with ten consecutively aspirated colonies was fixed with phosphate-buffered (pH 6.6) formalin-acetone for 30 seconds at 7 °C and subjected to cytochemical staining.

Each slide was triple stained, combining a double stain for specific and nonspecific esterases[26] and an eosinophilic granule stain with Biebrich scarlet[27] to analyze the lineages of the cells composing the colonies. For alpha-naphthyl butyrate esterase (a nonspecific esterase characteristic of the monocyte-macrophage lineage), the slide was covered with a substrate solution containing 1 mg/mL of Fast garnet GBC (Sigma Chemical Co, St Louis) for 15 minutes at room temperature and then washed with distilled water and air dried. For naphthol-ASD chloracetate esterase (a specific esterase characteristic of neutrophilic granulocytes), the slide was covered with a substrate solution containing 0.5 mg/mL of Fast blue RR (Sigma) for 20 minutes at room temperature and washed with distilled water and air dried. For eosinophilic granule stain with Biebrich scarlet (Sigma), the slide was dipped in a 0.1% Biebrich scarlet Tris-HCL (0.1 mol/L, pH 7.2) solution for two hours at room temperature. The slide was washed with distilled water, air dried, and mounted with glycerin gelatin. Using this triple stain, neutrophilic granulocytes from promyelocytes to segmented neutrophils appear as cells with discrete blue cytoplasmic staining (chloroacetate esterase), monocytes and macrophages have diffuse brown cytoplasmic staining (butyrate esterase), and eosinophils appear with coarse red granules (Biebrich scarlet).

In experiments for differentiation induction of leukemic cell lines by GM-CSF, poly-L-lysine-coated slides of pooled colony cells were subjected to the triple stain. In some experiments, single staining with Biebrich scarlet or Luxol fast blue[15,28] was also performed to detect eosinophilic differentiation. The cyanide-resistant myeloperoxidase reaction[26] was used to further confirm enzymatically the eosinophilic nature of Biebrich scarlet- and Luxol fast blue-positive cells.

Clonal Culture of Leukemic Cell Lines in Methylcellulose

HL-60 and KG-1 cells were plated at a final concentration of 3,000/mL and 10,000/mL, respectively, in Iscove's MEM containing 0.92% methylcellulose, 20% FBS, 10^{-4} mol/L alpha-thioglycerol, and 10% diluted GM-CSF. On day 7, colonies consisting of more than 20 cells were enumerated, and the smears of pooled colony cells were made and stained according to the method described previously for CFU-GM colonies.

RESULTS

Response of CFU-GM to Biosynthetic GM-CSF

GM-CSF dose response. Because of our previous results showing that preparations of natural GM-CSF partially purified from Mo-conditioned medium had no activity on mouse or rat bone marrow, all experiments were performed using human peripheral blood and bone marrow cells.[29,30] We determined the relationship of the number of CFU-GM colonies observed to the amount of GM-CSF added to two samples of normal light-density nonadherent bone marrow cells. As seen in Fig 1, the dose-response curve was sigmoid. Further studies using known molar concentrations of the GM-CSF sample (as determined by amino acid analysis) revealed that a 1:1,000 dilution was approximately equal to 250 pmol/L. Stimulation of colony growth was seen at concentrations as low as 2 pmol/L. At submaximal concentrations of GM-CSF, the colony sizes tended to be smaller along with the lower colony numbers. Control cultures without GM-CSF did not show any colony formation.

Cytochemical analysis of individual CFU-GM colonies. Colonies that developed at maximal stimulation with GM-CSF were analyzed using five separate bone marrow samples. The colonies were classified into four subtypes: pure neutrophilic granulocyte (G) colonies showing only chloroacetate esterase-positive cells, mixed granulocyte-macrophage (GM) colonies with a mixture of chloroacetate esterase-positive cells and butyrate esterase-positive cells, pure macrophage (M) colonies with only butyrate esterase-positive cells, and pure eosinophil (EO) colonies with only Biebrich scarlet-positive cells. In general, the sizes of the individual colonies varied from 20 to more than 300 cells.

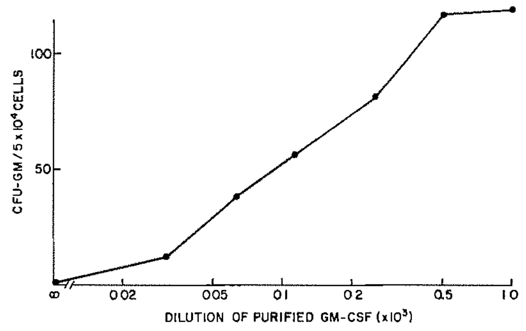

Fig 1. Biosynthetic GM-CSF dose response by normal human CFU-GM. Each point represents the mean value of two normal bone marrow samples. For each sample, triplicate cultures were performed at each dilution of GM-CSF. A 1:1,000 dilution of GM-CSF in this experiment was approximately equivalent to 250 pmol/L.

GM and M colonies tended to be larger than G and EO colonies. No unstained colonies were found. To obtain the distribution pattern of these cytochemically defined subtypes of colonies in each sample, 76 to 117 colonies were randomly aspirated and stained. Day 8 colonies were analyzed in all samples, and day 14 colonies in two samples. To avoid the development of fusion colonies, the number of light-density bone marrow cells plated was reduced to as low as 2.5×10^4/mL. This concentration usually yielded about 20 colonies per well. As shown in Table 1, the mean number of CFU-GM colonies formed was $102/5 \times 10^4$ cells. The four subtypes of colonies were consistently observed in all samples. G colonies predominated (70%) in all samples. GM colonies comprised 20%, and M and EO colonies about 5%.

The G colonies consisted of granulocytes of various maturational stages: promyelocytes and myelocytes being predominant, but band and segmented forms were consistently observed. Chloroacetate esterase–negative cells, indicative of immature blast cells, were not observed. GM colonies consisted of two types: one with a minor population of granulocytes among predominant monocytes-macrophages, and the other with almost equal numbers of cells of the two lineages. GM colonies with monocytes-macrophages as a minor population were rarely observed. The M colonies were usually composed of monocytes with moderate butyrate esterase activity, large macrophages with marked butyrate esterase activity, and intermediate cells showing maturational transition.[31] Rare M colonies, however, showed a pure population of monocytes that contained a few butyrate esterase–negative cells, probably representing immature monocytes such as monoblasts or promonocytes.[31]

The EO colonies showed substantial variability in frequency among the five samples. The lowest value of 0.9% observed in sample 5, however, increased to 12% in the day 14 analysis. The EO colonies usually consisted of 20 to 40 cells; mononuclear cells predominated, but a few bilobed forms also developed.

Two samples analyzed on day 14 exhibited a similar tendency in that the percentages of M and/or EO colonies became higher compared with those on day 8. Control culture without GM-CSF never yielded distinct colonies consisting of more than 20 cells in any sample.

Table 1. Cytochemical Analysis of Individual CFU-GM Colonies

	Bone Marrow Sample								
	1	2	3	4		5		Mean	
Culture Day	8	8	8	8	14	8	14	8	14
CFU-GM number per 5×10^4 cells	84	120	103	82	26	86	32	95	29
Colony number analyzed	72	111	103	110	24	117	40	102.6	32
Colony subtype (%)									
G	61 1	60 4	75 7	72 2	0	78 6	15 0	69 7	7 5
GM	31.9	27 0	10 8	13 6	8 3	17 9	25 0	20 2	16 6
M	4.2	5 4	4 8	6 8	54 2	2 6	47 5	4 7	50 9
EO	2 8	7 2	8 7	7 4	37 5	0 9	12 5	5 4	25 0

• The CFU-GM assay was performed at a concentration of GM-CSF (approximately 250 pmol/L), twofold above that required to give maximal stimulation of colony formation

Response of HL-60 and KG-1 Cell Lines to Biosynthetic GM-CSF

Although in control CFU-GM assays no detectable colonies were formed, the possibility remains that accessory cells such as a small number of phagocytic cells and lymphocytes in the light-density nonadherent bone marrow cell samples played some role in giving rise to colonies in GM-CSF–containing cultures.[32] This consideration led us to use HL-60 and KG-1 as homogeneous target cells. HL-60 may be induced to differentiate toward mature granulocytes and/or monocytes-macrophages by various compounds such as dimethyl sulfoxide, retinoic acid, tetradecanoyl phorbol ester, chemotherapeutic agents, and crude and purified lymphokines.[33-41] The KG-1 cell line is also known to be inducible toward macrophage differentiation by phorbol diesters and vitamin D derivatives.[40,41]

In HL-60, the liquid culture sample and unstimulated colony culture cells showed almost the same pattern of enzymatic expression: chloroacetate esterase–positive cells predominated and butyrate esterase–positive cells were negligible in number. However, a small proportion of cells was found to stain weakly with Biebrich scarlet stain. In stimulated cultures, the number of Biebrich scarlet–positive cells was consistently increased with increasing concentrations of GM-CSF (Table 2). The number of butyrate esterase–positive cells also increased. In contrast to the marked change in

Table 2. Response to GM-CSF of Human Myeloid Leukemia Cell Lines in Methylcellulose Culture

		Cytochemical Analysis of Pooled Colony Cells (%)			
Culture Condition	Colony Number	Negative*	Es-chl†	Es-b†	B-s†
HL-60					
Preculture	—	34 6	61.4	0	4 0
Unstimulated	$67 \pm 5/3 \times 10^2$	35.6	59.5	2.3	2 6
Stimulated					
Submaximal†	$82 \pm 6/3 \times 10^2$	33 7	52.0	6 0	8.3
Maximal‡	$150 \pm 14/3 \times 10^2$	39.6	32.1	16.3	12 0
KG-1					
Preculture	—	32.2	64 7	3.1	0
Unstimulated	$4 \pm 2/10^3$	42.0	56 0	2.0	0
Stimulated Maximal‡	$22 \pm 4/10^9$	35.2	61.5	3.3	0

Abbreviations Es-chl, naphthol-ASD chloroacetate esterase, Es-b, alpha-naphthyl butyrate esterase; B-s, Biebrich scarlet stain for eosinophil granules.

*Lacking any reaction to the triple stain.

†GM-CSF was added to give a final dilution of 1:8,000

‡GM-CSF was added to give a final dilution of 1:1,000 (Fig 1).

Each culture was performed in triplicate. Colony number is the mean ± SEM For cytochemical analysis of pooled colony cells, 300 cells were examined on a triple-stained smear, and the positive rate for each reaction was calculated

HL-60, KG-1 did not show a change in enzymatic pattern, and Biebrich scarlet–positive cells never appeared (Table 2).

To confirm the eosinophilic nature of the Biebrich scarlet–positive cells in the HL-60 cell line, Luxol fast blue and Wright-Giemsa staining was performed Distinct Luxol fast blue positivity and eosinophilic stain with Wright-Giemsa were recognized in almost the same percentages as with Biebrich scarlet stain. The percentage of cyanide-resistant peroxidase-positive cells in HL-60 cells stimulated with GM-CSF was also equal to that of Biebrich scarlet– and Luxol fast blue–positive cells.

Response of BFU-E to Biosynthetic GM-CSF

The effect of GM-CSF on erythroid burst formation was examined in cultures containing low concentrations (0.33 U/mL) of EPO (Fig 2). No enhancement of erythroid bursts was seen in cultures containing amounts of GM-CSF shown to be stimulatory on CFU-GM (Fig 2B). This observation is in contrast to the consistent burst formation in the standard EPA- and EPO-containing cultures (Fig 2A). The addition of GM-CSF to the standard EPA- and EPO-containing cultures did not enhance BFU-E numbers, but rather caused a measurable decrease in cloning efficiency (Fig 2C).

DISCUSSION

Purified biosynthetic (recombinant) GM-CSF produced by COS monkey kidney cells transfected with a cDNA clone–encoding human GM-CSF caused consistent stimulation of normal CFU-GM proliferation and differentiation. The GM-CSF dose-response curve was typically sigmoid, and in several experiments we have seen no inhibitory effect at concentrations up to 1 nmol/L. All of the three G, GM, and M colony types were stimulated, as well as eosinophil colonies. G colonies predominated in day 8 cultures, most becoming diffuse and losing colony configuration by day 14. In contrast, M and EO and some of the GM colonies continued their growth to reach larger-colony sizes during the second week of incubation. The resultant colony number was considerably decreased on day 14. This difference in the rate of colony formation may reflect the kinetic characteristics of each CFU-GM subpopulation in response to GM-CSF. It remains to be seen whether the GM-CSF can stimulate all CFU-GM or a specific subset. Theoretically, purification of all human CSFs is a prerequisite for such a study.

The presence in the light-density nonadherent bone marrow cell sample of accessory cells capable of releasing CSFs[30] makes it difficult to draw unambiguously the conclusion that GM-CSF has direct activities on CFU-GM and CFU-EO. An ideal experimental system would require single cells capable of differentiation to granulocytes/macrophages and eosinophils in the presence of pure CSF. To examine the direct actions of GM-CSF, we chose human myeloid leukemia cell lines as readily available homogeneous target cells for GM-CSF. Both HL-60 and KG-1 responded to GM-CSF by increasing their proliferative rates in liquid culture (data not shown) and plating efficiencies in methylcellulose culture.

Cytochemical examination of GM-CSF–stimulated HL-60 cells and KG-1 cells showed that HL-60 can be induced to differentiate towards monocytic and eosinophilic lineages. Some of the stimulated HL-60 cells became fully mature eosinophils or macrophages. Eosinophilic granules stained with Biebrich scarlet or Luxol fast blue were also positive for cyanide-resistant peroxidase, confirming the eosinophilic nature of these HL-60 cells. As the unstimulated cultures of HL-60 contained small proportions of Biebrich scarlet–positive cells, the stem cells of this cell line appear to have a multipotential ability to differentiate toward monocytic and eosinophilic as well as neutrophilic lineages when properly stimulated. Induced differentiation of the HL-60 cell line has been extensively investigated;[33–41] however, there are only a few published analyses of eosinophilic differentiation.[42–44] Lu et al observed an increase of eosinophilic colonies after 14 days of methylcellulose culture stimulated by crude sources of CSF.[42] In contrast, Metcalf did not note such an increase in agar culture stimulated by GM-CSFα.[43] In the present study, the total increase in the number of eosinophilic cells and the appearance of more mature forms with confluent large granules were apparent. In contrast to HL-60, the enhanced proliferation of the KG-1 cell line by GM-CSF was not accompanied by differentiation induction. The consistent differentiation induction of HL-60 by GM-CSF toward monocytic and eosinophilic lineages is compatible with the findings obtained in the CFU-GM assay of normal hematopoietic progenitor cells.

In the present study, we were unable to detect basophilic colonies from normal bone marrow samples or basophilic differentiation in GM-CSF–stimulated HL-60 cells. We stained 100 colonies from sample 4 and pooled HL-60 colony cells with toluidine blue No colony nor HL-60 cells showed a distinct metachromasia for this dye, suggesting that basophil stimulation is not a functional activity of the GM-CSF. However, there still remains a possibility that CFU-Baso was present at a rate lower than 1% of the CFU-GM colonies and missed from the present aspiration analysis.

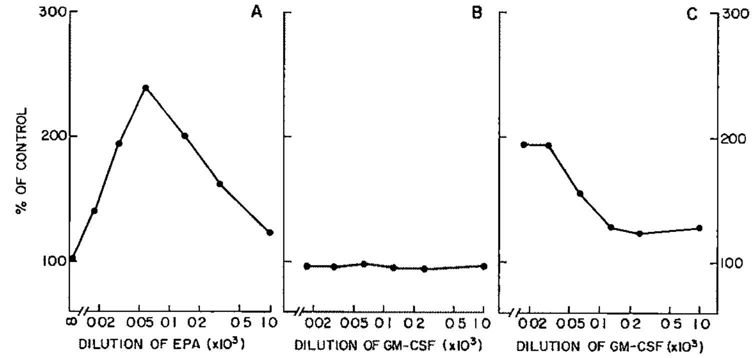

Fig 2. Response to biosynthetic GM-CSF by normal human BFU-E. (A) EPA dose response of BFU-E at an EPO concentration of 0.33 U/mL. (B) GM-CSF dose response of BFU-E in a culture containing EPO (0.33 U/mL) only. (C) GM-CSF dose response of BFU-E in a culture containing EPA (× 16,000 dilution:peak stimulation in Panel A) and EPO (0.33 U/mL). The mean number of BFU-E obtained in the control cultures (from two different donors) with only EPO (0.33 U/mL) were 17 and 55 per well (3.3 × 10^4 cells in 0.1 mL). These values were defined as 100% in each experiment. Each point represents the mean value of two normal peripheral blood buffy coat samples assayed in duplicate. A 1:1,000 dilution of GM-CSF in these experiments was approximately equivalent to 250 pmol/L.

It has been reported that purified murine GM-CSF is capable of stimulating BFU-E. Metcalf et al showed by the sequential addition of purified GM-CSF, a burst-promoting activity (BPA) and EPO in a single hematopoietic progenitor cell culture assay, that the GM-CSF can stimulate the initial divisions of BFU-E, although it did not induce terminal maturation of BFU-E leading to EPO responsiveness.[45] Our observations using simultaneous addition of human biosynthetic GM-CSF to BFU-E assays containing EPA and EPO or EPO alone did not result in stimulation of BFU-E Since the addition of large amounts of GM-CSF to the EPO plus EPA assay caused a detectable inhibition of burst formation, some unidentifiable interactions between these stimulators and progenitor or accessory cells are suggested. In this context, others using the same recombinant GM-CSF sample observed a consistent stimulatory activity on BFU-E growth (David Nathan and Colin Sieff, Dana-Farber Cancer Institute, Harvard Medical Society, personal communication, July 1985). Further experiments are required to elucidate the interactions of EPA, EPO, and GM-CSF on human BFU-E. Human GM-CSF also differs from murine multi-CSF (IL-3), which exerts stimulatory activity on multiple progenitors of erythroid,[46] megakaryocyte,[47] eosinophil,[47] and mast cell[48] lineages as well as on granulocyte and monocyte lineages.

It is apparent that comparative studies on the biologic differences between human GM-CSF and other CSFs purified to homogeneity from various sources are needed. Such comparisons, along with genetic structure analyses, will clarify how many kinds of human CSF exist and their biologic roles in the control of human hematopoiesis. Based on the present observations, we can conclude that T cell-derived human GM-CSF is stimulatory to CFU-GM and CFU-EO

NOTE ADDED IN PROOF

Biosynthetic GM-CSF stimulated the growth of human BFU-E in the presence of erythropoietin when the precursor cells were enriched by removing adherent, phagocytic, and E-rosetting cells from the buffy coat.

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Biologic Properties In Vitro of a Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor

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Recombinant human granulocyte-macrophage colony-stimulating factor (rH GM-CSF) was purified to homogeneity from medium conditioned by COS cells transfected with a cloned human GM-CSF cDNA and shown to be an effective proliferative stimulus in human marrow cultures for GM and eosinophil colony formation. The specific activity of purified rH GM-CSF in human marrow cultures was calculated to be at least 4 × 10^7 U/mg protein. Clone transfer experiments showed that this proliferation was due to direct stimulation of responding clonogenic cells. Acting alone, rH GM-CSF did not stimulate erythroid colony formation, but in combination with erythropoietin, increased erythroid and multipotential colony formation in cultures of peripheral blood cells. rH GM-CSF had no proliferative effects on adult or fetal murine hematopoietic cells, did not induce differentiation in murine myelomonocytic WEHI-3B cells, and was unable to stimulate the survival or proliferation of murine hematopoietic cell lines dependent on murine multi-CSF (IL 3). rH GM-CSF stimulated antibody-dependent cytolysis of tumor cells by both mature human neutrophils and eosinophils and increased eosinophil autofluorescence and phagocytosis by neutrophils. From a comparison of these effects with those of semipurified preparations of human CSFα and -β, it was concluded that rH GM-CSF exhibited all the biologic activities previously noted for CSFα.

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THE granulocyte-macrophage colony-stimulating factors (CSFs) are specific glycoproteins that control the production and functional activity of granulocytes and macrophages. Four murine CSFs[1] and one human-active GM-CSF[2,3] have been purified to homogeneity and some of their biologic actions determined. Complementary DNAs (cDNA) for two murine CSFs (GM-CSF[4] and Multi-CSF[5,6] [IL-3]) have been cloned and the biologic activity of their translation products in monkey COS cells shown to be identical to that of the corresponding CSFs purified from murine sources.[7-9]

Recently, a cDNA for a human-active GM-CSF has been cloned from a library prepared from the human Mo T-leukemia cell line, and analysis showed a 70% homology in the nucleotide sequence of the protein-coding region with murine GM-CSF cDNA.[2] GM-CSF with the same *N*-terminal amino acid sequence was independently purified from Mo-conditioned medium[2,3] and shown to stimulate granulocyte-macrophage colony formation by human cells. Both native and recombinant GM-CSFs were also shown to be active on mature human polymorphs, inhibiting their migration [2,10]

Previous biochemical studies have indicated the presence of at least two distinct forms of CSF able to stimulate granulocyte and macrophage colony formation by human cells in media conditioned by normal human placenta,[11] the human GCT tumor cell line,[12] and the U5637 bladder cancer cell line.[13] These have been termed CSFα and CSFβ[11] and have been shown to differ in their range of progenitor target cells. Preparations containing CSFα have been observed to stimulate eosinophil colony formation, but preparations containing CSFβ do not.[11] Human CSFα is inactive on mouse cells, but CSFβ resembles murine G-CSF biochemically, is a good proliferative stimulus for murine granulopoietic cells, is able to induce differentiation in murine myeloid leukemia cells, and competes with murine G-CSF for receptors on both murine and human cells.[13]

These studies were undertaken to determine whether the present human GM-CSF cDNA codes for the production of a GM-CSF of an α or β type or a novel type of CSF with other properties. CSF was produced by transfection of monkey COS cells with the human GM-CSF cDNA, and the CSF secreted by these cells was purified by separative protein chemistry. A series of assays was performed using this purified recombinant CSF to determine its range of functional activities.

MATERIALS AND METHODS

Preparation and Purification of rH GM-CSF

The cDNA for human GM-CSF was cloned from a cDNA library constructed using messenger RNA (mRNA) from the Mo human T-leukemia cell line by screening candidate clones in a direct expression system using COS cells To prepare rH GM-CSF for testing, mass cultures of COS cells in cell factories (Nunc, Roskilde, Denmark) were transfected with cloned human GM-CSF cDNA in the p 91203 (B) vector. The final medium fed to the cells was serum free, and after four days of further incubation the medium was harvested, concentrated by ultrafiltration, and the GM-CSF purified to homogeneity as previously described.[2,3] Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretic analysis of the purified GM-CSF revealed a major silver-staining band of 19 kilodaltons (kD) with several very faint bands in the 16 to 19 kD range. From bovine serum albumin (BSA) standards, the protein content of the purified material prepared for biologic testing was estimated to be 20 μg/mL.

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Assays on Recombinant Human GM-CSF

The purified recombinant human GM-CSF preparation (rH GM-CSF) was diluted 1:100 in 10% fetal calf serum (FCS)-saline, and serial dilutions of 0.1 mL were added to the cultures for assay

Human marrow cultures. Marrow and peripheral blood samples were obtained with the prior informed consent of patients according to a protocol approved by the Ethics Committee of the Walter and Eliza Hall Institute. Fifty thousand unfractionated human bone marrow cells from patients with no significant hematologic disease were cultured in 35-mm Petri dishes using 1 mL of Dulbecco's modified Eagle's medium and a final concentration of 20% FCS and 0.3% agar. Cultures were incubated for up to 14 days at 37 °C in a fully humidified atmosphere of 10% CO_2 in air.

Colony formation (clones of more than 40 cells) was scored at ×35 magnifications using a dissection microscope after seven and 14 days of incubation. To determine the cellular composition of the resulting colonies, whole cultures were fixed using 2.5% glutaraldehyde and methanol and, after drying, stained with Luxol fast blue/hematoxylin. Colony classification was performed at ×400 magnifications.

Units of colony-stimulating activity were calculated from the linear portion of the dose-response curve assigning 50 U/mL to the concentration stimulating the formation of 50% of the maximal colony numbers.

The human placental–conditioned medium (HPCM) stage II used in these experiments was prepared as described previously.[11] The semipurified CSFα and -β fractions were obtained using phenyl-Sepharose chromatography of medium conditioned by the human bladder cancer cell line U5637, using methods described previously.[11]

Human peripheral blood cultures A total of 2×10^5 nonadherent peripheral blood cells from β-thalassemia patients were cultured in agar medium with or without the addition of 1.5 units of semipurified human urinary erythropoietin using Iscove's modified Dulbecco's medium and 25% FCS. Cultures were scored after 14 days of incubation and, after fixation, stained with benzidine/hematoxylin for colony classification.

Murine assays. Volumes of 0.1 mL of serial dilutions of rH GM-CSF were added to agar cultures of 75,000 C57BL adult marrow cells in 20% FCS or 20,000 12-day CBA fetal liver cells in 20% human plasma. Control cultures contained 400 units of purified murine GM-CSF, Multi-CSF, G-CSF, M-CSF,[1] or saline. Colony formation was scored after seven days of incubation. To determine whether rH GM-CSF could initiate but not sustain colony formation by murine hematopoietic cells, 400 units of Multi-CSF were added after two days of incubation to replicate cultures of fetal liver cells, and the cultures were scored after a total incubation period of seven days. After scoring, cultures were stained with Luxol fast blue/hematoxylin and colonies classified according to cellular composition.

Microwell assays using the continuous hematopoietic cell lines 32D cl.3 (kindly supplied by Dr J. Greenberger, Harvard Medical School, Boston) and FCD-P1 (kindly supplied by Dr T.M. Dexter, Paterson Laboratories, Manchester) were performed in microtiter trays using 200 cells per well in 10 μL of Dulbecco's modified Eagle's medium with 20% FCS. Serial dilutions of 5 μL of each test sample were added and viable cell counts performed after 48 hours of incubation.

To determine whether rH GM-CSF was able to induce differentiation in WEHI-3B D^+ myelomonocytic leukemic colonies, 0.1 mL of serial dilutions of rH GM-CSF were added to agar cultures of 300 WEHI-3B D^+ cells. The percentage of colonies exhibiting differentiation was determined after seven days of incubation.[14] Control cultures contained 0.1 mL of serial dilutions of serum from C57BL mice injected three hours previously with 5 μg of endotoxin that contained G-CSF or semipurified human CSFα and -β.

Clone Transfers

Sequential clones developing in marrow or peripheral blood cultures after four to eight days of stimulation by rH GM-CSF were removed intact using a fine Pasteur pipette of a small enough diameter to ensure a minimal amount of surrounding agar and no contaminating cells in the surrounding agar

Clones were placed on the surface of recipient agar cultures containing no cells but either 0.1 mL saline, 0.1 mL rH GM-CSF, 0.1 mL HPCM, or for peripheral blood clones, 1.5 units of erythropoietin or 1.5 units of erythropoietin plus 0.1 mL rH GM-CSF. The concentrations of rH GM-CSF and HPCM used in the recipient cultures were 200 or 400 U/mL. Five clones were transferred to each recipient culture, a procedure ensuring a subsequent 1:500-fold dilution of any stimulus carried over in the agar droplets surrounding the clone.[15] The cell content of the clones was recorded immediately after transfer. After a further six to ten days of incubation, the size of transferred clones was again recorded and the clones placed on microscope slides and stained either with Luxol fast blue/hematoxylin or benzidine/hematoxylin.[1]

Functional Activation of Mature Cells

Mature neutrophils and eosinophils were fractionated from normal human peripheral blood using a hypertonic metrizamide gradient as described previously.[16]

Antibody-dependent cytolysis of tumor cells. These assays were performed using rabbit IgG antidinitrophenyl (anti-DNP) antibodies and tripitrophenyl (TNP)-coupled mouse P815 mastocytoma cells as described previously.[17] The incubation time used was 2.5 hours.

Autofluorescence of eosinophils. After an incubation period of one hour, autofluorescence of eosinophils was monitored by fluorescence-activated cell sorting (FACS) analysis at 460 nm as described previously.[18]

Phagocytosis. Human peripheral blood neutrophils, prepared as previously mentioned, were suspended at 20×10^6 cells per mL in phosphate-buffered saline (PBS). Dried bakers' yeast (Tandaco, Melbourne) was suspended in PBS so that optical density (OD) 540 equaled 1.6

The rH GM-CSF in medium/FCS was diluted in buffer (8.0 g NaCl, 0 2 g KCl, 1 15 g Na_2HPO_4, 0.2 g $MgCl_2 \cdot 6H_20$, 0.11 g $CaCl_2$, and 0.4 g glucose per liter of distilled water, pH 7.4). The final concentration of the rH GM-CSF in the assay was 2 ng/mL.

Assays were performed in triplicate in Wasserman tubes kept at 4 °C until the addition of the following CSF in buffer, 50 μL; yeast, 250 μL; fresh autologous serum, 100 μL; and cells, 100 μL Tubes were incubated for one hour at 37 °C, placed on ice, centrifuged at 4 °C, and then the cells were resuspended in 50 μL cold PBS. Smears were prepared, fixed in methanol, and stained with Giemsa. At least 200 cells were scored per preparation, counting the number of phagocytosed yeast organisms. Total counts were pooled for the triplicate CSF and buffer control preparations, compared by χ^2 analysis, and expressed as percentages.

Binding Competition Assays

The preparation of radioiodinated G-CSF and the binding assay to WEHI-3B D^+ cells have been described in detail elsewhere.[13] Briefly, 1×10^6 WEHI-3B D^+ in 120 μL of Dulbecco's modified Eagle's medium containing 10% (vol/vol) FCS were incubated with 150,000 cpm of ^{125}I G-CSF with or without a 20-fold excess of unlabeled G-CSF or varying concentrations of the test competitor at 37 °C for one hour. Cell-bound radioactivity was separated from unbound radioactivity by centrifugation of cells through FCS and cell pellets counted on a LKB Autogamma gamma counter (Uppsala, Sweden).

RESULTS

Colony Formation Stimulated by rH GM-CSF in Cultures of Human Marrow Cells

To minimize the risk of missing direct or indirect effects of rH GM-CSF, initial assays were performed using serial dilutions of purified recombinant material in unfractionated cultures of 50,000 bone marrow cells from patients without significant hematologic disease. Control cultures were stimulated by unfractionated human placental–conditioned medium or semipurified fractions of CSFα and -β. The recombinant material exhibited colony-stimulating activity in agar cultures of all 14 marrow samples tested, and a typical example of the results obtained is shown in Fig 1. From this titration curve it was calculated that the colony-stimulating activity of the rH GM-CSF preparation was 4 × 10^7 U/mg. In the example shown in Fig 1, the recombinant material stimulated plateau numbers of both day 7 and day 14 colonies at a concentration of 5 ng/mL, and with progressive reduction of this concentration, a typical sigmoid dose-response curve was obtained, the lowest concentration stimulating detectable colony formation being approximately 300 pg/mL. With the 14 marrow samples analyzed in detail, individual marrows varied up to fourfold in their responsiveness to stimulation by the recombinant material, and with some marrow samples, maximal colony formation was observed with a concentration of 1 ng/mL, giving an estimate of specific activity of 1.6 × 10^8 U/mg.

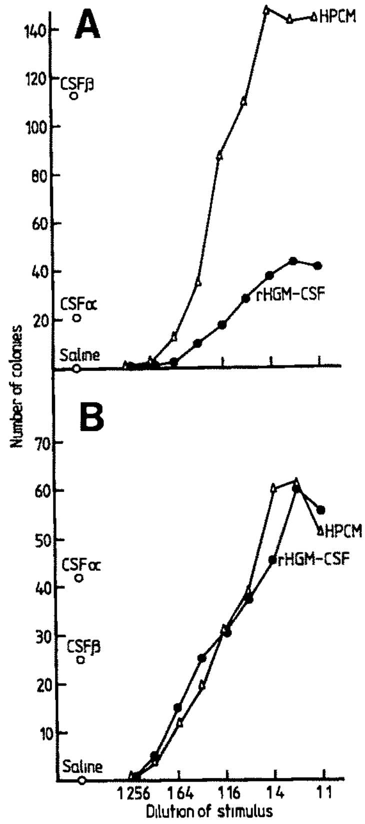

Fig 1. Colony formation at days 7 (A) and 14 (B) in cultures of 50,000 human marrow cells stimulated by 0.1 mL of serial dilutions of a preparation containing 200 ng/mL purified rH GM-CSF. Control cultures contained 0.1 mL of serial dilutions of stage II HPCM or semipurified preparations of CSFα and CSFβ. Note the rise with time in the number of colonies in cultures stimulated by rH GM-CSF.

For the 14 marrow samples analyzed, colony counts at seven days with the various stimuli were as follows: rH GM-CSF 41 ± 30; HPCM, 140 ± 110; CSFα, 27 ± 34; and CSFβ, 138 ± 77 (±SD). At 14 days the following counts were recorded: rH GM-CSF, 85 ± 55; HPCM, 103 ± 96; CSFα, 54 ± 40; and CSFβ, 37 ± 35 (±SD). It will be seen that using rH GM-CSF colony counts rose between seven and 14 days, a characteristic of CSFα but not CSFβ, and individual examples of these trends are shown in Fig 1 and Table 1. A characteristic feature of the cultures stimulated by rH GM-CSF was the relatively slow onset of the development of colonies, a feature also seen in cultures stimulated by CSFα-containing preparations and contrasting with the early onset and early disappearance of colony formation in cultures stimulated by CSFβ.[19]

As shown in the example in Fig 1, the number of colonies stimulated by rH GM-CSF was usually lower than that stimulated by the HPCM control preparation. HPCM contains both CSFα and -β, and there is evidence that neither CSFα nor -β, acting alone, is able to stimulate all available progenitor cells.[20] The colony counts in cultures stimulated by rH GM-CSF therefore suggested that the recombinant material was not able to stimulate the proliferation of all available granulocyte-macrophage progenitors.

Morphologic analysis of day 7 colonies stimulated by rH GM-CSF showed that these were granulocytic, granulocyte-macrophage, and monocyte-macrophage colonies together with a few small eosinophil colonies (Table 1). In the cultures stimulated by rH GM-CSF, the percentage of monocyte-macrophage colonies was higher than in control cultures stimulated by HPCM. This pattern persisted at 14 days, with the most frequent colony type again being monocyte-macrophage. It should be noted that day 14 cultures stimulated by rH GM-CSF always contained prominent eosinophil colonies. In control cultures stimulated by CSFα, a similar pattern of colony formation was observed to that in cultures stimulated by rH GM-CSF, whereas in cultures stimulated by CSFβ, eosinophil colonies never developed.

Studies on the effects of rH GM-CSF were made on cultures of marrow cells from three untreated patients with acute myeloid leukemia. Cells from one of these (M2) failed to proliferate when stimulated either by HPCM or rH GM-CSF. Cells from the other two patients (M2 and M1) formed small clusters (two to 40 cells) when stimulated either by HPCM or rH GM-CSF. In both cases, the frequency (690 and 700/10^5 cells with 400 units rH GM-CSF and 640 and 650/10^5 cells with 400 units HPCM) and size of clusters developing were equivalent.

Effects of rH GM-CSF on Erythroid and Multipotential Colony Formation

No erythroid or multipotential colonies developed in agar cultures of bone marrow or peripheral blood cells stimulated by rH GM-CSF alone. However, addition of rH GM-CSF to cultures of nonadherent peripheral blood cells containing 1.5

Table 1. Morphologic Types of Colonies in Human Marrow Cultures After Seven and 14 Days of Stimulation by Human Recombinant GM-CSF

Incubation Time	Marrow Sample No.	Stimulus	Mean Number of Colonies	Percentage of Colonies			
				G	GM	M	Eosin
7 days	1	saline	0	—	—	—	—
		HPCM	143	62	34	4	0
		rH GM-CSF	41	10	65	25	0
		CSFα	21	13	56	31	0
		CSFβ	113	45	44	11	0
	2	saline	4	0	29	71	0
		HPCM	127	35	48	17	0
		rH GM-CSF	115	7	42	46	5
14 days	1	saline	2	0	0	100	0
		HPCM	57	11	34	43	12
		rH GM-CSF	54	0	41	40	19
		CSFα	42	2	45	38	15
		CSFβ	26	11	56	33	0
	2	saline	4	0	0	100	0
		HPCM	33	34	26	34	6
		rH GM-CSF	99	4	31	44	21

Abbreviations: G, neutrophilic granulocyte; GM, neutrophilic granulocyte-monocyte; M, monocyte-macrophage; Eosin, eosinophil.

A total of 5 × 10^4 human marrow cells were cultured for seven or 14 days. The various stimuli (HPCM, rH GM-CSF, and CSFα and -β fractionated from U5637-conditioned medium) were all used at a concentration fourfold in excess of that stimulating maximal colony numbers to develop. The mean colony counts are from duplicate cultures. After colony counts were performed, cultures were fixed and stained with Luxol fast blue/hematoxylin, and differential colony counts were performed on the entire colonies in each culture.

units of erythropoietin consistently resulted in the formation of two- to threefold higher numbers of burst erythroid colonies than in cultures containing only erythropoietin and in the development of small numbers of multipotential colonies containing erythroid cells. Two examples of this effect are shown in Table 2, and in two other experiments similar results were obtained. A similar pattern of colony formation was observed in cultures stimulated by a mixture of HPCM and erythropoietin and in cultures containing semipurified CSFα and erythropoietin. In contrast, the combination of semipurified CSFβ with erythropoietin did not lead to an enhanced erythroid colony formation.

Titration experiments showed that enhanced erythroid colony formation could be observed with dilutions of rH GM-CSF over the same range as those able to stimulate granulocyte-macrophage colony formation by direct action.

Table 2. Enhancement of Erythroid and Multipotential Colony Formation by Recombinant Human GM-CSF

Experiment No	Stimulus	Erythropoietin	Mean Number of Colonies		
			Erythroid	Multipotential	Nonerythroid
1	saline	−	0	0	0
		+	13 ± 2	0	1 ± 1
	HPCM	−	0	0	68 ± 8
		+	55 ± 6	2 ± 1	52 ± 12
	CSFα	−	0	0	36 ± 6
		+	36 ± 3	2 ± 2	39 ± 7
	CSFβ	−	0	0	32 ± 3
		+	8 ± 5	0	19 ± 5
	rGM-CSF	−	0	0	38 ± 7
		+	36 ± 6	2 ± 2	40 ± 7
2	saline	−	0	0	3 ± 1
		+	11 ± 1	0	2 ± 2
	HPCM	−	0	0	133 ± 9
		+	29 ± 3	1 ± 1	148 ± 9
	CSFα	−	0	0	51 ± 9
		+	17 ± 8	1 ± 1	46 ± 10
	CSFβ	−	0	0	109 ± 14
		+	12 ± 3	0	94 ± 9
	rGM-CSF	−	0	0	35 ± 10
		+	18 ± 3	1 ± 1	41 ± 9

A total of 2 × 10^6 nonadherent peripheral blood cells were cultured in 1-mL agar cultures containing 400 units of test stimulus with or without 1.5 units of semipurified human urinary erythropoietin. Cultures were scored after 14 days of incubation. Mean colony counts are ± SD of four replicate cultures.

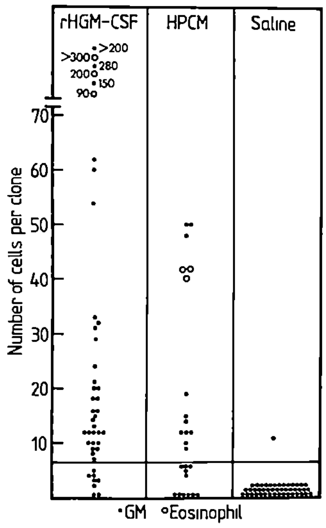

Fig 2. Behavior of four-day clones initiated by rH GM-CSF after transfer to recipient cultures containing rH GM-CSF, HPCM, or saline. The concentration of rH GM-CSF and HPCM in recipient cultures was 400 U/mL. The line indicates the mean size of clones at transfer. Note the progressive size increase in the clones after ten further days of incubation in cultures containing rH GM-CSF or HPCM.

Direct v Indirect Effects of rH GM-CSF

To determine whether the colony-stimulating effects of rH GM-CSF were the result of direct action on responding clonogenic cells, cultures were initiated using 50,000 marrow cells and 400 units of rH GM-CSF. At four days, individual clones containing four to ten cells (mean size, six cells) were removed using a fine pipette and transferred intact with minimal surrounding agar to the surface of recipient agar cultures containing 400 units of rH GM-CSF, 400 units of HPCM, or 0.1 mL saline but no cells. Analysis of the fate of these transplanted clones after a further ten days of incubation (Fig 2) showed that clone size increased progressively in recipient cultures containing both rH GM-CSF and HPCM, whereas clones died when transferred to cultures lacking CSF. Clones exhibiting progressive proliferation with continued stimulation by rH GM-CSF included both granulocyte-macrophage and eosinophil colonies, indicating that rH GM-CSF was able to act directly on responding clones of both types.

In contrast, 150 sequentially sampled clones initiated by 200 units of rH GM-CSF for five, six, or eight days in cultures of peripheral blood (clone size, three to 60 cells) generated only a single erythroid, one mixed erythroid colony, and one mixed neutrophil-eosinophil colony when transferred to cultures containing either erythropoietin or rH GM-CSF plus erythropoietin (Table 3). These results suggested that most of the enhanced erythroid colony formation seen when rH GM-CSF was combined with erythropoietin was not ascribable to an ability of rH GM-CSF, acting alone, to initiate the proliferation of some BFU-E by direct action, a property that has been documented for murine GM-CSF acting on murine BFU-E.[21]

Inactivity of rH GM-CSF on Murine Cells

Recombinant human GM-CSF failed to stimulate any type of colony or cluster formation in cultures of 75,000 C57BL bone marrow cells or 20,000 12-day CBA fetal liver cells. In cultures initiated using rH GM-CSF to which 400 units of murine Multi-CSF were added after two days of incubation, again no colonies or clusters were seen after a total incubation period of seven days. As shown in Table 4, these negative results were similar to those obtained with CSFα and were in contrast to the ability of CSFβ-containing preparations to stimulate granulocyte colony formation in cultures of adult mouse bone marrow or fetal liver cells and the weak but reproducible ability of CSFβ to initiate erythroid and multipotential colony formation when stimulation of colony formation was completed by the delayed addition of murine Multi-CSF after two days of incubation

In microwell assays using the 32D murine hematopoietic cell line (responding to murine Multi-CSF) and the murine FDC-P1 cell line (responding to both murine Multi-CSF and murine GM-CSF),[22] rH GM-CSF failed to support the survival or stimulate the proliferation of cells of either line.

Table 3. Development of Erythroid and Nonerythroid Colonies From Transfered Clones Initiated in Peripheral Blood Cultures Using rH GM-CSF

Day of Clone Transfer	Stimulus in Recipient Cultures	Number of Colonies					
		G	GM	M	Eosin	E	Mixed
5	saline	2	0	1	2	0	0
	rH GM-CSF	0	0	0	5	0	0
	rH GM-CSF + Epo	2	0	0	7	0	0
6	saline	1	0	0	1	0	0
	rH GM-CSF	1	0	0	7	0	1
	rH GM-CSF + Epo	0	0	0	6	0	1
8	saline	0	1	0	0	0	0
	Epo	1	0	0	0	1	0
	rH GM-CSF + Epo	0	0	0	5	0	0

Abbreviations. E, burst erythroid, Mixed, one colony of neutrophils and eosinophils, one colony of neutrophils, eosinophils, and erythroid cells; Epo, erythropoietin.

Clones were initiated for five, six, or eight days in cultures of 2 × 10^5 nonadherent peripheral blood cells using 200 units of rH GM-CSF. Twenty-five sequential clones of three to 60 cells were transfered to each type of recipient culture. Recipient agar cultures contained 0.1 mL saline, 200 units rH GM-CSF, and/or 1.5 units erythropoietin. Cultures were scored after a total incubation period of 14 days.

Table 4. Effect of rH GM-CSF in Cultures of Mouse Fetal Liver Cells

Stimulus	Number of Colonies Nonerythroid	 Pure or Mixed Erythroid
Cultures without addition of murine multi-CSF		
Saline	0	0
MLCM	34 ± 8	0
SCM	74 ± 25	15 ± 3
HPCM	47 ± 4	0
CSFα	2 ± 2	0
CSFβ	14 ± 1	0
rH GM-CSF	0	0
Delayed addition of murine multi-CSF on day 2		
Saline	7 ± 2	0.3 ± 0.3
MLCM	69 ± 6	10 ± 2
HPCM	85 ± 4	6 ± 4
CSFα	15 ± 9	0.5 ± 0.5
CSFβ	57 ± 18	3 ± 2
rH GM-CSF	6 ± 2	0

Abbreviations: MLCM, mouse lung-conditioned medium; SCM, pokeweed mitogen-stimulated mouse spleen-conditioned medium

Twenty thousand 12-day CBA fetal liver cells were cultured in 1-mL agar cultures containing 20% human plasma. Cultures were scored after seven days of incubation. Data are colony counts ± SD from four replicate cultures. CSFα- and -β-containing preparations were from U5637 bladder cancer-conditioned medium.

In agar cultures of 300 WEHI-3B D^+ myelomonocytic leukemic cells, rH GM-CSF and CSFα failed to induce differentiation in the leukemic colonies whereas, as previously documented,[13] CSFβ fractions exhibited high differentiation-inducing activity (Fig 3).

Binding Competition Studies

Human CSFβ is able to competitively block binding of ^{125}I-labeled murine G-CSF to either murine or human cells, whereas human CSFα has no such capacity.[13] As shown in Fig 4, rH GM-CSF had no capacity to block binding of ^{125}I G-CSF to 10^6 WEHI-3B D^+ murine myelomonocytic leukemia cells even at a concentration of 100,000 U/mL when coincubated at 37 °C for one hour.

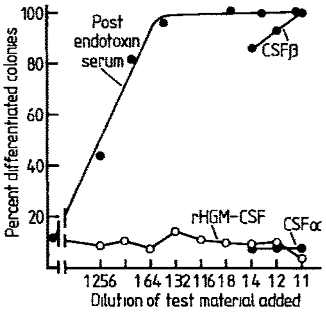

Fig 3. Failure of rH GM-CSF to induce differentiation in WEHI-3B D^+ murine myelomonocytic leukemia colonies. CSFα was also inactive, but CSFβ preparations induced differentiation comparable to that in control cultures containing postendotoxin mouse serum as a source of murine G-CSF.[23]

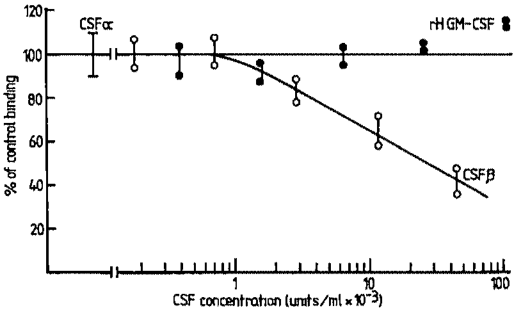

Fig 4. Failure of rH GM-CSF to block binding of ^{125}I-labeled murine G-CSF to WEHI-3B D^+ myelomonocytic leukemic cells. Note that human CSFβ competes effectively but that CSFα, like rH GM-CSF, has no competitive action (the bar indicates the labeling level in the presence of CSFα).

Stimulation of Mature Neutrophils and Eosinophils by rH GM-CSF

To determine whether rH GM-CSF was able to increase the functional activity of mature human neutrophils and eosinophils, populations of peripheral blood neutrophils (97% pure) and eosinophils (92% pure) were tested for their capacity to exhibit increased antibody-dependent lysis of ^{51}Cr-labeled tumor cells when incubated with rH GM-CSF' during the 2.5-hour assay. Control preparations were incubated with semipurified preparations of CSFα and CSFβ.

As shown in Table 5, rH GM-CSF significantly increased the lysis of tumor cells by both neutrophils and eosinophils, as did CSFα. Titration of the rH GM-CSF indicated that the 50% end point in this elevated response occurred with a concentration of approximately 60 pg/mL, a tenfold lower concentration than that usually stimulating 50% maximal colony formation. In agreement with previous observations,[17] preparations containing CSFβ had no effect on eosinophil-mediated killing and only stimulated neutrophil-mediated killing.

CSFα, but not CSFβ, has previously been shown to increase eosinophil autofluorescence.[18] As shown in Table 6,

Table 5. Stimulation by rH GM-CSF of Antibody-Dependent Cytolysis of ^{51}Cr-Labeled P815 Mastocytoma Cells by Human Eosinophils and Neutrophils

Stimulus	Percentage of Cytotoxicity* Eosinophils (92% pure)	 Neutrophils (97% pure)
Saline	0.7†	2.1
rH GM-CSF	39.5	11.9
CSFα	22.9	12.7
CSFβ	0.2	10.8

*There was no significant release of ^{51}Cr in the absence of antibody by target cells.

†Arithmetic mean of triplicate determinations. The eosinophil cytotoxicity stimulated by rH GM-CSF and CSFα was significantly higher ($P < .001$) than that stimulated by CSFβ or exhibited by control cells to which saline had been added. The neutrophil cytotoxicity stimulated by rH GM-CSF, CSFα, and -β was significantly higher ($P < .001$) than that of unstimulated cells.

Table 6. Ability of rH GM-CSF to Increase Autofluorescence of Human Eosinophils

Stimulus	Percentage of Increased Autofluorescence *v* Control Cells*
rH GM-CSF	36 ± 16
CSFα	37 ± 12
CSFβ	−1 ± 13

*Calculated from FACS analysis above an arbitrary channel number.[18]

rH GM-CSF at a concentration of 2 ng/mL had the same ability to increase the autofluorescence of eosinophils.

As shown in Table 7, incubation of purified neutrophils in the presence of 2 ng/mL rH GM-CSF for one hour significantly increased their capacity to phagocytose yeast organisms

DISCUSSION

In agreement with the original biologic monitoring experiments[2] undertaken during the cloning of the cDNA for human GM-CSF, the present experiments have indicated that rH GM-CSF, as synthesized by monkey COS cells, is an effective proliferative stimulus for human granulocyte-macrophage progenitor cells leading to typical colony formation in semisolid cultures. In addition, however, the present experiments have shown that rH GM-CSF is also a highly effective proliferative stimulus for human eosinophil colony formation. Plateau levels of granulocyte-macrophage and eosinophil colonies were stimulated by a concentration of 1 to 5 ng/mL rH GM-CSF, and minimum colony formation was observable with 300 pg/mL. The overall level of colony-stimulating activity in the purified material was calculated as at least 4 × 10^7 U/mg, a figure in agreement with the previous estimate[2] and reasonably similar to the specific activity of the four purified murine granulocyte-macrophage CSFs in bone marrow cultures, which vary from 10^8 to 10^9 U/mg.[1] The rH GM-CSF exhibited somewhat stronger stimulating activity for monocyte-macrophage colony formation than for granulocyte colony formation. This, coupled with the fact that the plateau numbers of colonies, particularly at day 7, were always lower than with HPCM, suggests that rH GM-CSF is not able to stimulate the proliferation of all GM progenitors and may in fact stimulate the same subset as stimulated by CSFα.

A prominent feature of the action of rH GM-CSF was the stimulation of eosinophil colony formation. Eosinophil colony formation has previously been observed in cultures stimulated by semipurified CSFα preparations,[11] but an alternative possible explanation of these earlier observations was that the eosinophil colony formation was due to the presence in the preparations of a contaminating eosinophil CSF. The present results with purified GM-CSF of recombinant origin clearly indicate that this molecule possesses the capacity to stimulate eosinophil colony formation and suggests that the previously observed activity of CSFα-containing preparations was not due to a contaminating eosinophil CSF.

Table 7. Stimulation by rH GM-CSF of Phagocytosis of Yeast Organisms by Peripheral Blood Neutrophils

Incubated With	Number of Cells Scored	Percentage of Cells With Indicated Numbers of Yeast Organisms					
		0	1	2	3	4	≥5
Buffer	600	56	15	11	10	5	3
rH GM-CSF	600	22	22	24	15	11	6

The rH GM-CSF was used at a final concentraton of 2 ng/mL. The incubation time was one hour. The results are significantly different by χ^2 analysis ($P < .001$).

No erythroid colonies were observed in cultures stimulated by rH GM-CSF. However, combined stimulation by rH GM-CSF and erythropoietin of nonadherent peripheral blood cells led to the formation of increased numbers of pure burst-erythroid colonies and occasional multipotential colonies containing erythroid cells.

Analysis using clone transfers indicated that the actions of rH GM-CSF on granulocyte-macrophage and eosinophil colony formation were direct effects on the CSF, acting alone, on responding clonogenic cells. However, transfer experiments using clones initiated by rH GM-CSF showed that most of the enhanced erythroid colony formation in cultures containing rH GM-CSF and erythropoietin seemed not to be ascribable to a direct action of rH GM-CSF in initiating proliferation of BFU-E. Thus the situation with rH GM-CSF differs from that documented previously for murine GM-CSF in which it was possible to demonstrate a direct action of GM-CSF on a major fraction of murine BFU-E.[21] The basis for the observed erythroid enhancement requires further investigation. Two possibilities needing investigation are (1) that rH GM-CSF and erythropoietin need to act simultaneously on BFU-E or (2) that the action of rH GM-CSF may be indirect and be mediated by stimulation of some other cell type in the culture to produce an erythroid-enhancing factor.

Tests using mature human neutrophils and eosinophils showed that rH GM-CSF was an effective stimulus of antibody-dependent cytotoxicity for tumor target cells, increased phagocytic activity by neutrophils, and increased eosinophil autofluorescence—general properties noted previously for human-active CSFs.[17,18] These observations on the effects of rH GM-CSF on mature end cells are in agreement with a recent study[10] indicating that this molecule, as purified from Mo cell–conditioned medium, has an ability to act on mature neutrophils as monitored by the inhibition of neutrophil migration.

The rH GM-CSF had no capacity to stimulate the proliferation of murine hematopoietic cells from either marrow or fetal liver cells or of cells of two murine continuous cell lines responding to murine Multi-CSF or GM-CSF. Furthermore, no differentiation-inducing activity was noted when rH GM-CSF was added to cultures of the murine myelomonocytic cell line WEHI-3B.

A summary of the observed biologic activity in parallel assays of rH GM-CSF compared wtih those of semipurified human CSFα and -β is presented in Table 8. It is clear that the activities of rH GM-CSF differ completely from those of CSFβ-containing material, and recent studies have in fact shown that murine G-CSF, not GM-CSF, is the functional analogue of human CSFβ.[13] The activities of rH GM-CSF

Table 8. Comparison of Range of Biologic Activities of rH GM-CSF, Human CSFα and Human CSFβ

Biologic Activity	Type of Human CSF		
	rH GM-CSF	CSFα	CSFβ
Stimulation of			
7D GM colonies	+	+	++
14D GM colonies	++	++	+
Eosinophil colonies	++	++	0
Potentiation of erythroid and multipotential colonies	+	+	0
Stimulation of murine GM colonies	0	0	++
Initiation of murine erythroid and mixed colonies	0	0	+
Induction of differentiation in murine leukemic cells	0	0	++
Binding competition with murine G-CSF	0	0	++
Stimulation of mature cell function			
Neutrophil	++	++	++
Eosinophil	++	++	0

are identical to those of CSFα-containing preparations, and it can be concluded provisionally that rH GM-CSF is CSFα. Verification of this conclusion should be possible since GM-CSF with the same *N*-terminal sequence has been purified from Mo cell–conditioned medium,[2,3] and the properties of this purified native molecule need to be determined using the aforementioned broad range of assay systems.

The purification has been reported of a human pluripoietin from medium conditioned by the bladder cancer cell line, U5637.[24] From its reported ability to stimulate erythroid, multipotential, granulocyte-macrophage and to induce differentiation in murine myeloid leukemia cells, this pluripoietin does not resemble CSFα, CSFβ or rH GM-CSF. Furthermore, if it has any homology with murine multi-CSF at the amino acid sequence level, it would be unlikely to have homology with rH GM-CSF since there is no homology between murine GM-CSF and multi-CSF.[4] Therefore, despite the ability of rH GM-CSF to promote erythroid colony formation and the formation of some mixed erythroid colonies, it is unlikely that rH GM-CSF is a candidate human analogue of murine multi-CSF. In this context, murine GM-CSF also potentiates erythroid and mixed colony formation, in this case by being able to initiate (but not sustain) the proliferation of erythroid and multipotential precursors.[21] Previous studies have shown that murine GM-CSF is also able to stimulate eosinophil colony formation by adult and particularly fetal progenitor cells.[25,26] Thus, the ability of rH GM-CSF to stimulate the proliferation of human eosinophil precursors does not make it different in principle from its murine analogue.

Purified rH GM-CSF should be an effective agent for routine use in stimulating colony and cluster formation in cultures of human normal cells and in stimulating the functional activation of mature human neutrophils and eosinophils. Subject to more extensive testing, this agent may also be a useful proliferative stimulus for use in cultures of myeloid leukemic cells. However, from the present data using normal granulocyte-macrophage progenitor populations, it needs to be emphasized that rH GM-CSF is likely not to be able to stimulate the proliferation of all available progenitor cells. To achieve this latter result it will be necessary to produce recombinant human CSFβ and, if additional human CSFs exist, recombinant versions of these and to use a mixture of such recombinant CSFs for routine use so that all available progenitor cells are able to be stimulated.

If recombinant GM-CSF material with equivalent biologic activity to the present material can be generated using yeast or bacterial expression systems, the prospects are good that it will become possible to test the in vivo actions of this regulator in clinical situations when there is a need either to stimulate increased cell production or to stimulate the functional activity of existing mature cells.

ACKNOWLEDGMENT

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Sickle Cell Anemia Patients Have Low Erythropoietin Levels for Their Degree of Anemia

By Judith B. Sherwood, Eugene Goldwasser, Robert Chilcote, L. David Carmichael, and Ronald L. Nagel

We have studied serum immunoreactive erythropoietin (SIE) levels in 28 patients with sickle cell anemia (SCA) without renal insufficiency and in 17 patients with nonhemoglobinopathy anemias of comparable severity using a sensitive radioimmunoassay procedure. An exponential relationship between SIE level and degree of anemia was noted in all patients. However, in nonhemoglobinopathy anemia, a sharp rise in the SIE level occurred as hemoglobin (Hb) levels fell below about 12 g/dL, whereas in sickle cell patients the increase was not marked until hemoglobin fell to about 9 g/dL. The response was more blunted in older SCA patients than in younger ones. A linear regression model relating SIE level to Hb level, presence/absence of SCA, and age explained 63% of the variation in SIE. We conclude that the serum erythropoietin levels in SCA increased at a lower hemoglobin concentration and are of a lower magnitude than that of the other anemias.

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A CONSIDERABLE NUMBER of clinical and experimental observations demonstrate the inverse relationship between the erythropoietin level in plasma and urine and the level of tissue oxygenation.[1] A direct correlation between elevated erythropoietin levels and the degree of hypoxic stimulus has been shown[2,3] as well as suppression of erythropoietin levels with hyperoxia[4] or red cell plethora.[5] The inverse relationship between the red cell mass and hemoglobin concentration, and the circulating erythropoietin titer, has been amply demonstrated.[1,6]

The erythropoietin response in several anemias has been more difficult to establish, in part because of the lack of precision of the bioassay methods heretofore available and because of parameters of the specific anemia such as $Hb\text{-}O_2$ affinity, renal damage, etc.

We have studied the correlation between erythropoietin levels and hemoglobin concentrations in patients with sickle cell anemia who have red cells with low oxygen affinity as well as in patients with anemias not associated with hemoglobinopathies. We have used the sensitive and specific radioimmunoassay for erythropoietin,[7] which allows the accurate measurement of normal and depressed levels of human serum erythropoietin without prior concentration of samples. Finally, we have examined the effect of age on the Hb level–erythropoietin relationship.

MATERIALS AND METHODS

Subjects. Serum was obtained from patients with anemias of various causes. 28 patients had sickle cell disease (ten adults and 18 children and young adults). Two additional subjects with $S\beta^+$-thal were also studied. Of the 18 children and young adults with sickle cell disease, 12 were males and six females, and they had a mean age of 9.2 ± 5.5 years with a range of 1 to 17. Their mean hemoglobin level was 7.6 ± 1.6 g/dL with a range of 3.1 to 10.1 g/dL; the reticulocyte count was 19.4% ± 10.1% with a range of 0.1% to 36.3%. All patients had a BUN less than 20 mg/dL. Of the ten adult patients with sickle cell anemia, seven were females and three males; they had a mean age of 31.0 ± 6.6 years with a range of 22 to 40 years. Thirteen patients had anemias not associated with hemolysis nor with any hemoglobinopathy; three patients had chronic lymphocytic leukemia, one had chronic myelocytic leukemia, four had multiple myeloma, and five had anemias not associated with hematologic malignancy. All of the patients with non–sickle cell anemia had normal BUN levels (mean, 16.1 ± 3.42 mg/dL) and serum creatinine levels less than 2 mg/dL. Four patients had hemolytic anemias not associated with hemoglobinopathy. None of these patients had been transfused and all had a BUN less than 20 mg/dL. The two pediatric patients with $S\beta^+$-thal had a mean age of 7 ± 2 years, a mean hemoglobin level of 7.6 ± 0.65 g/dL, a reticulocyte count of 15.2% ± 4.8%, a mean corpuscular volume (MCV) of 71.5 ± 0.5 μm^3, a mean corpuscular hemoglobin (MCH) of 24.8 ± 0.45 pg, a mean corpuscular hemoglobin concentration (MCHC) of 35% ± 0.95%, and a bilirubin level of 0.7 ± 0.7 mg/dL. All hematologic and other laboratory data of these patients are shown in Table 1. Blood was allowed to clot on ice for two hours. The serum was separated by centrifugation and stored at −20 °C until assay.

Assay procedures. Serum immunoreactive erythropoietin (SIE) was measured in each serum sample by using the radioimmunossay procedure described by Sherwood and Goldwasser.[7] Erythropoietin was labeled with ^{125}I by using the water-insoluble oxidizing agent Iodogen (Pierce Chemical Co, Rockville Ill).[8] The iodinated tracer was incubated with erythropoietin standard or 100 μL of serum sample and with antiserum in a phosphate buffer diluent. The antiserum was used at a dilution that gave a bound-to-total ^{125}I-erythropoietin ratio (B/T) between 0.20 to 0.30. The antibody-erythropoietin complex was separated from the free hormone either by precipitation with ammonium sulfate by using a modification of the method developed by Stanley[9] or by use of the second antibody technique previously described.[7]

Statistical analysis. Preliminary examination of the data revealed a nonlinear relationship of the SIE level to varying degrees of anemia, with nonuniform variances proportional to the mean SIE level (Table 1). Therefore, a log-linear model with a nonzero asymptote of the form $\log(SIE_i - C) = b_0 + b_1(Hb_i) + e_i$ was chosen, and the other patient variables (age, presence of sickle cell disease, and other hematologic characteristics) were evaluated for their contribution by standard regression analysis.[10] The asymptote c was set at 10 mU/mL, approximating the normal mean −2 SD as established in this laboratory's assay. This procedure allowed us to select variables that significantly affected the SIE level, with approximately normal distribution of error terms and an F value for the model of 22.0 ($P < 2 \times 10^{-8}$).

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Table 1. Laboratory Values of Anemic Patients

Anemia	No of Patients	Hb (g/dL)	Hct (%)	Retic (%)	HbF (%)	MCV (μm^3)	MCH (pg)	MCHC (%)	Bilirubin (mg/dL)	EPO (mU/mL serum)
Sickle Cell Anemia										
9 2 ± 5 5 yr	18	7 61 ± 1 58	21 14 ± 4 71	19 35 ± 10 1	6 48 ± 6 8	90 17 ± 10 5	32.95 ± 5 5	36 26 ± 2 8	2 97 ± 2 8	192 52 ± 291 7
(1–17)		(3 1–10 1)	(8–27 5)	(0–36 3)	(0–20 3)	(69–108)	(23 5–41 5)	(33 2–44 8)	(0–8 8)	(26 9–1300)
31 ± 6 6 yr	10	8 08 ± 1 46	14 1 ± 2.9	10 3 ± 4 4	5 2 ± 3 2	95 8 ± 9 6	32 9 ± 2 5	34 3 ± 2 2	3 2 ± 1 6	56 ± 63
(22–40)		(6 0–10 8)	(6 2–28)	(4 1–16)	(2 8–13 4)	(86 7–111)	(28 5–35 2)	(31 3–37.4)	(1 7–4.3)	(12–220)
Nonhemolytic, Nonhemoglobinopathy	13	9 9 ± 2	28 5 ± 5 7	—	—	93 6 ± 19 1	32.3 ± 6 5	34 6 ± 1 9	2 ± 3 5	420 ± 365
		(6 2–12 8)	(18–34 8)			(49 4–12 6)	(16 9–44 1)	(31 9–38 6)	(0 2–10 4)	(21–1300)
Hemolytic, Nonhemoglobinopathy	4	8 88 ± 3 92	26 40 ± 11 4	32.17 ± 30 3	—	—	—	—	—	395 0 ± 195.0
		(3 2–11 9)	(9 4–33)	(8.9–66 4)						(220–610)

Abbreviations: Hct, hematocrit; EPO, erythropoietin; Hb, hemoglobin

Values are expressed as mean ± SD. Numbers in parentheses are the range of values. Hematologic indexes (MCV, MCH, MCHC) were obtained by the Coulter Counter (Coulter Electronics, Hialeah, Fla). Erythropoietin values were determined in the radioimmunoassay.

RESULTS

SIE levels were measured in 17 patients with anemias of various causes not associated with sickle cell anemia, other hemoglobinopathies, or renal disease (including four patients with hemolytic anemia) and in 28 patients with sickle cell anemia (ten adults and 18 children); of these, the 17 non–sickle cell anemia patients and 26 sickle cell anemia patients for whom all variables were recorded were used in the analysis. The two patients with Sβ^+-thal were analyzed separately. We found that Hb level, age, and sickle cell anemia status predicted 63% (R^2) of the variance in SIE (Figs 1 and 2), with $F_{3,39} = 22.0$ ($P < 2 \times 10^{-8}$). The significance of the contribution of the individual terms was tested, and presence/absence of sickle cell anemia was highly significant ($P < .0004$); the effect of age, however, was significant only among sickle cell anemia patients ($P \cong .011$). The adult sickle cell anemia patient with 220 mU/mL of SIE appeared to be an outlier; however, there were no major alterations in the model when this patient was excluded, and there appeared to be no technical reasons to question the accuracy of the SIE determination.

The mean SIE level of the anemic patients was significantly higher than the normal value of 21 ± 6 ($\bar{x}$ ± SD) mU/mL previously found with this radioimmunoassay.[7,11] The 17 patients with nonhemoglobinopathy-associated anemias had the highest levels: 414 ± 327 mU/mL of serum (range, 21 to 1,300 mU/mL), with a mean hemoglobin concentration of 9.7 ± 2.5 g/dL (range, 6.1 to 12.8 g/dL). The mean SIE level of the four patients with hemolytic anemias not associated with hemoglobinopathy was 395 ± 195 mU/mL (range, 220 to 610 mU/mL), a value that did not differ significantly from the levels found in the other nonhemoglobinopathy anemias (Fig 1). The mean value found for the patients with sickle cell anemia was 144 ± 234 mU/mL of serum (range 12 to 1,300 mU/mL), with a mean hemoglobin concentration of 7.8 ± 1.5 g/dL (range, 3 to 11 g/dL). As shown in Fig 2, the sickle cell anemia patients have less SIE at any level of hemoglobin; SIE levels rise only when considerably lower Hb levels are reached, as compared with non–sickle cell anemia patients.

The adult sickle cell anemia patients had a mean SIE level of 56 ± 63 mU/mL of serum (range, 12 to 220 mU/mL)

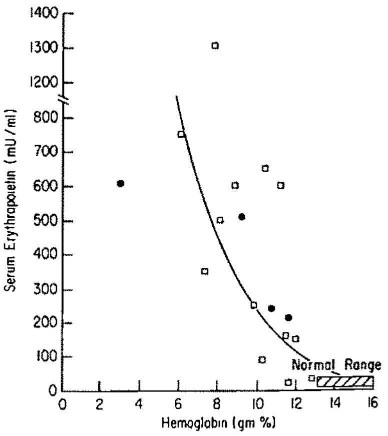

Fig 1. Erythropoietin levels in nonhemoglobinopathy anemias for 13 patients with nonhemolytic (□) and four patients with autoimmune hemolytic (●) anemia. The line represents the regression equation log (SIE − 10) = 8.91 − 0.35 (Hb), and the crosshatched box indicates the normal range.

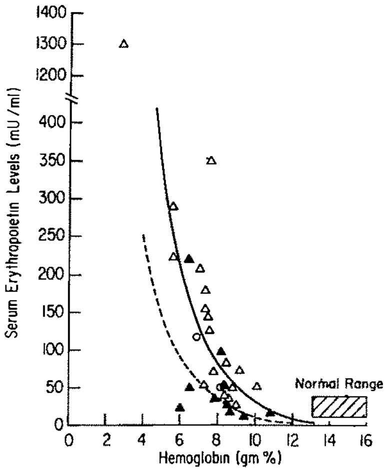

Fig 2. Erythropoietin levels in sickle cell anemia for 18 pediatric (△) and ten adult (▲) patients; two children with Sβ^+-thal disease (O) are also shown. The regression equation log (SIE − 10) = 8.91 − 0.54 (Hb) − 0.04 (age) is shown for the mean age of the two age groups.

with a mean hemoglobin concentration of 8.1 ± 1.5 g/dL (range, 6 to 11 g/dL). The children with sickle cell anemia, with a similar hemoglobin concentration of 7.6 ± 1.6 g/dL (range, 3 to 10 g/dL), had a mean SIE level of 193 ± 292 mU/mL of serum (range, 27 to 1,300 mU/ml). When a child with 3 g/dL hemoglobin and an erythropoietin level of 1,300 mU/mL was excluded, the mean SIE level for the children was 127 ± 96 mU/mL, and the mean hemoglobin level was 7.9 ± 1.1 g/dL. Analysis of covariance comparing the adult and pediatric sickle cell patients showed that the average erythropoietin level was significantly different at $P < .02$, even when the child with the very low hemoglobin level (3.1 g/dL) and high erythropoietin level (1,300 mU/mL) was not included in the computation.

The two children with $S\beta^+$-thal had a mean Hb level of 7.6 ± 0.65 g/dL and an SIE level of 84.2 ± 33.8 mU/mL (50.5, 118). When they were included in the regression model, there was no significant alteration in its parameters, and its strength actually increased slightly ($R^2 = 65\%$)

DISCUSSION

SIE levels were determined in pediatric and adult patients with sickle cell disease and in patients with nonhemoglobinopathy anemias of various causes, including hemolytic anemias by using our sensitive radioimmunoassay procedure.[7] An exponential relationship between the SIE level and the hemoglobin concentration was noted in all patients. In patients with nonhemoglobinopathy anemias, we observed a drastically increased slope at a hemoglobin concentration of approximately 11 g/dL or less (Figs 1 and 2). This hemoglobin level can be considered as a threshold. The data presented here on the inverse relationship between hemoglobin concentration and immunoreactive erythropoietin levels in anemias other than sickle cell (Fig 1) are in general agreement with the previously published data based on biologic activity.[12]

The relationship between Hb levels and SIE in the sickle cell anemia patients departs from this since these patients exhibit elevated levels of erythropoietin only at a hemoglobin concentration below about 9 g/dL (Fig 2). The patients with nonhemoglobinopathy-associated anemias and other types of hemolytic anemias do not exhibit this hemoglobin threshold and have levels of SIE that are greater than that of the sickle cell patients (Fig. 2). Our observation that patients with sickle cell anemia produce less immunoreactive erythropoietin at a given hemoglobin concentration than do patients with nonhemoglobinopathy anemias (hemolytic and nonhemolytic) agrees with a previous observation using the biologic assay.[13]

We conclude that the relationship between SIE and Hb levels in sickle cell anemia can be described by an exponential curve that is significantly displaced to the left. In addition, the level of SIE reached by these patients is also significantly lower than that observed in other anemias. Analysis of the variables involved in the relationship between SIE and Hb levels by using a linear regression model indicated that age, Hb level, and presence or absence of anemia explained 63% (R^2) of the variance in the SIE level. Of special interest is the effect of age, as pediatric sickle cell anemia patients tended to have significantly higher SIE levels than adults. The potential mechanism for the low SIE levels in sickle cell disease include interference with the renal synthesis of erythropoietin and the displacement to the right of the O_2 equilibrium curve.

The well-known right shift in the oxygen equilibrium curve would facilitate oxygen delivery to the tissues, decreasing the hypoxic burden of a given level of anemia. Sickle cells have a higher p50 because of the intrinsically lower affinity for oxygen of the hemoglobin S polymer[14] and because of the increase in intracellular 2,3-diphosphoglyceric acid levels.[15] In addition, kinetic factors (decreased uptake and diffusion of O_2) contribute to this anomaly in vivo.[16]

Renal tissue damage in these patients might compromise the synthesis of erythropoietin or the hypoxia sensor presumed to be located in this organ. The renal damage, which differs from renal insufficiency, involves a concentration defect, progressive vascular damage particularly to the medulla, and structural abnormalities of the glomeruli and tubules.[17] In favor of this interpretation is the age dependency of the level of SIE in sickle cell anemia patients (Fig 2). Individuals with chronic renal failure have SIE levels that are higher than normal, but lower than expected for the degree of anemia.[18] In addition, Morgan et al[13] observed that in adults with sickle cell anemia the concentration of erythropoietin was correlated positively with creatinine clearance and inversely with the BUN and that hemoglobin concentrations were depressed in the presence of minor degrees of renal insufficiency. Further work is necessary to establish definitively the mechanism of the decreased erythropoietin response in the patient with sickle cell anemia.

ACKNOWLEDGMENT

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A Monoclonal Antibody Reactive With Human Eosinophils

By Hiroshi Saito, Kazuyoshi Yamada, Jacqueline Bréard, Osamu Yoshie, and Georges Mathé

EO-1, an IgGI murine monoclonal antibody raised against human eosinophilic leukemia cells, reacts with eosinophils, basophils, platelets, and a few (2%) mononuclear cells but not with neutrophils. In the bone marrow, mature and immature eosinophils and basophils express the EO-1 antigen, whereas immature myeloid cells do not. The distribution of EO-1 antigen on leukemic cells is concordant with this finding, ie, typical myeloid lines (HL-60, KG-1, and ML-1) and fresh acute myelogenous leukemia cells are all unreactive with EO-1. Immunoprecipitation of an extract from surface-^{131}I-labeled platelets with EO-1 and sodium dodecyl sulfate polyacrylamide gel electrophoresis, under reducing or nonreducing conditions, yielded a specific band of molecular weight 23,000. Previously described monoclonal antibodies reacting with eosinophils also recognize neutrophils. EO-1 is a unique antibody with specificity restricted to eosinophils, basophils, and platelets and might therefore be a valuable reagent for the study of their function and differentiation.

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MANY MONOCLONAL antibodies that react with peripheral blood (PB) granulocytes have been reported. Some of these, PM-81, FMC10, and 3G8,[1–4] react with both neutrophils and eosinophils, whereas others are specific only for neutrophils (My-1, IB5, 4D1).[5,6] Antibodies such as B37.3[7] recognize neutrophils and platelets, whereas ANH7 is specific for neutrophils, eosinophils, and basophils.[8] Thus far there has been no report of a monoclonal antibody that reacts with eosinophils but not with neutrophils. Such an antibody would be a useful reagent for analyzing eosinophil function and differentiation. This report concerns the generation of such an antibody, termed EO-1.

MATERIALS AND METHODS

Production of monoclonal antibody The EO-1 cell line was established by the fusion of NS-1 myeloma cells with spleen cells from Balb/c mice immunized with eosinophils purified from a patient with eosinophilic leukemia by using the method of Köhler and Milstein[9] as previously described.[10]

Isolation of peripheral mononuclear cells and spleen cells Peripheral mononuclear cells (MNC) and spleen cells were isolated by Ficoll-metrizoate (F/M) (Lymphoprep, Nyegaard, Sweden) density gradient centrifugation. The T cell fraction (E+, cells forming rosettes with sheep erythrocytes) and T cell depleted fraction (E−, cells not forming rosettes with sheep erythrocytes) were prepared as described previously.[10] Monocytes were obtained by adherence to plastic dishes pretreated with fetal calf serum.[11]

Isolation of eosinophils, basophils, and neutrophils Erythrocytes were separated from whole PB by sedimentation in 30% Plasmasteril (Fresenius, Hamburg, Germany), and the buffy coat was collected. The separation of eosinophils and neutrophils was carried out according to the procedure described by Ellis et al[12] with a slight modification. The buffy coat cells were spun on a Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) discontinuous density gradient, using four different concentrations of Percoll (56%, 64%, 68%, and 77%). The neutrophils were collected on the 68% Percoll layer and eosinophils on the 77% layer. A cell fraction relatively enriched in basophils was obtained on top of the 64% Percoll layer In all cases, purity was assessed by morphology or reactivity with the lineage-specific monoclonal antibodies OKT3 (Ortho Pharmaceutical Corp, Raritan, NJ) B1, I2, Mo1, Mo2 (Coulter, Hialeah, Fla), HNK-1 (Becton Dickinson), IF10,[10] and surface immunoglobulin (SmIg). J5 antibodies were kindly provided by Dr J Ritz (Harvard University, Boston). Platelets were prepared by centrifugation of whole blood for ten minutes at 250 g.

Flow cytofluorimetric analysis A total of 10^6 cells were incubated with EO-1 antibody at 4 °C for 30 minutes, washed twice in phosphate buffered saline (PBS) containing 0 1% bovine serum albumin and 0.05% sodium azide, and incubated with 25 μL of fluorescein-conjugated F(ab')$_2$ goat antimouse immunoglobulin (Cappel Laboratories, Cochranville, Pa) for 30 minutes at 4 °C. The cells were then washed and suspended in 1 mL of PBS. Stained cells were analyzed with a cell sorter (CS-2,000, Showa Denko, Tokyo). The intensity of fluorescence per cell was recorded by a multiple-channel pulse-height analysis. As a control for all experiments, a murine monoclonal IgGI antibody that specifically reacts with a cytoplasmic antigen was used

Cell fractionation by the immune rosette technique In some experiments, cells were separated into immune rosette-positive and -negative fractions as previously described.[10] Briefly, F/M MNC or bone marrow (BM) cells were incubated with specific antibody for 30 minutes on ice and washed three times with PBS. Ox or sheep erythrocytes coupled with Fab-antimouse immunoglobulin antibody (IgG) (Cappel Laboratories) by the chromium chloride method[13] were added to these suspensions. The mixture was centrifuged at 400 g for five minutes. The percentage and morphology of rosette-forming cells were analyzed. This method was used for the identification of cells reacting with the EO-1 antibody and also for the preparation of lymphocyte subsets.

Human leukemic cell lines. Cell lines (T cell lines: CEM, JM, MOLT4F, HSB, HPB; B cell lines: DAUDI, RAJI, P3HR1, BALL-1, RPMI 8866, SERAFINA, RAEL, ARH77; Pre-B or null cell lines: NALM-1, NALM-6, NALM16, Reh; Myeloid-monocytic lines: K562, HEL, HL-60, KG-1, ML-1, ML-2, THP-1, U937) were maintained in suspension culture as previously described.[10]

Human leukemic cells. Leukemic cells from PB or BM from patients with acute lymphocytic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), CML–blastic crisis (CML-BC), basophilic leukemia, and Burkitt's lymphoma were tested for reactivity with EO-1.

Characterization of EO-1 antigen. Cell surface antigens of intact platelets isolated from a healthy donor were radioiodinated by the lactoperoxidase technique using carrier-free Na^{131}I (2 mCi, Amersham Corp, Oakville, Canada)[14] The cell membranes were solubilized for 30 minutes in a lysis buffer (PBS containing 0 5% Nonidet P-40 [Sigma Chemical Co, St Louis]) and 1 mmol/L phenylmethylsulfonyl fluoride on ice, and the lysate was centrifuged

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at 10,000 rpm for five minutes. Radiolabeled extracts were precleaned by incubation with protein A–Sepharose 4B(Pharmacia) for four hours. The cleared extract was incubated with 10 μL of monoclonal antibody (ascites fluid) of EO-1 or control ascites for one hour at 4 °C and then incubated with protein A–Sepharose 4B overnight at 4 °C. The gels were then washed three times with PBS, and the immune complex was eluted with the sodium dodecyl sulfate (SDS) sample buffer for electrophoresis. The eluates were boiled for two minutes and electrophoretically analyzed by 10% SDS polyacrylamide gel electrophoresis (PAGE) according to the methods of Laemmli.[15] The gels were stained, dried, and subjected to autoradiography.

RESULTS

Selection of monoclonal antibody. One supernatant EO-1 that reacted with eosinophils but not with neutrophils was selected. EO-1 cells were cloned by a limiting dilution, and ascites were generated. The immunoglobulin class of EO-1 antibody was determined by the Ouchterlony method as IgG1.

Cytofluorimetric analysis. The fluorescence intensity profile showed intense staining of the majority of eosinophils, and the percentage of positive eosinophils by the cell sorter was concordant with that calculated by the standard morphologic method. As shown in Figs 1A and 1B, the higher the content of eosinophils in the fraction, the more prominent were the cells positive with EO-1. The majority of neutrophils did not react with EO-1. The small number of MNC in the PB seemed to be positive with EO-1 (Fig 1C), and a small fraction of BM cells also reacted with EO-1 (Fig 1D). Monoclonal antibody used for the control did not show any reaction.

Reactivity of EO-1 with eosinophils and basophils by the immune rosette method. Eosinophils prepared by Percoll fractionation from normal PB were tested for their reactivity

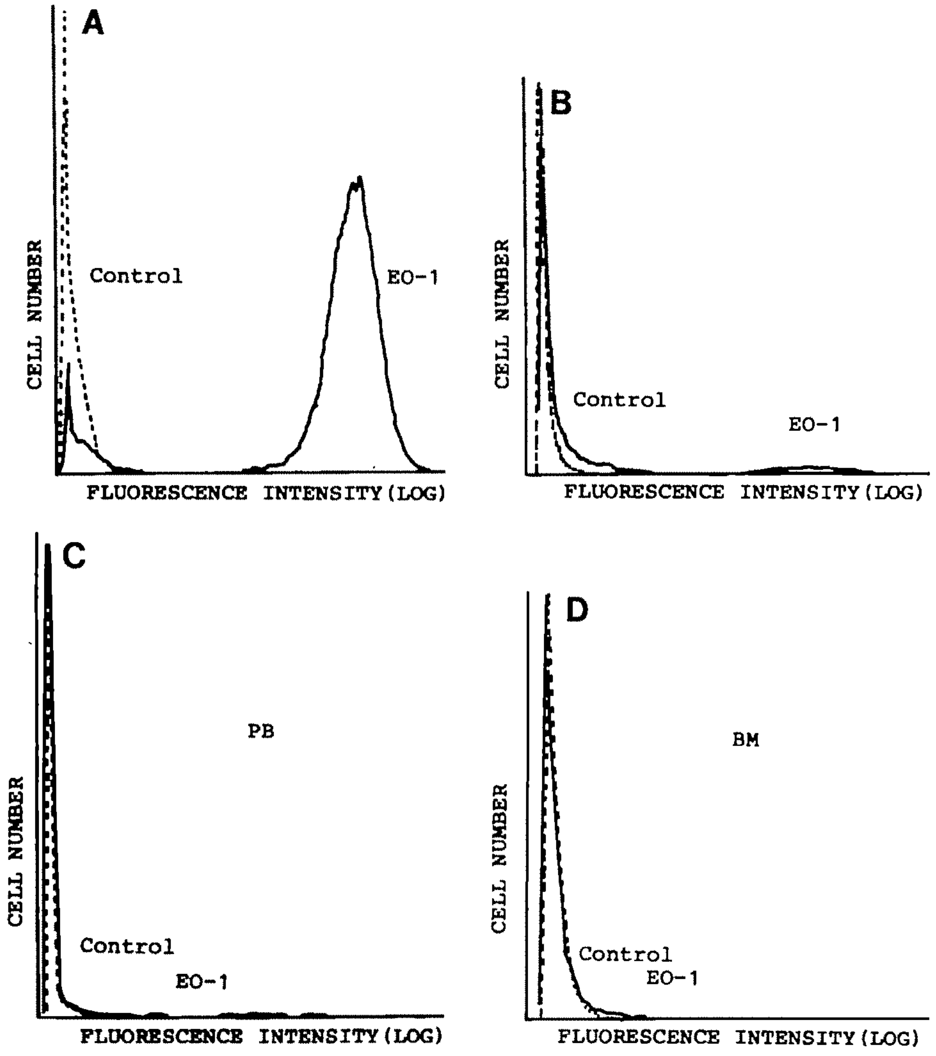

Fig 1. Reactivity of EO-1 on various cell fractions from (A) eosinophils purified from PB by Percoll gradient (77%) (purity: eosinophils, 94%; neutrophils, 6%), (B) neutrophils purified from PB by Percoll gradient (66%) (purity: neutrophils, 87%; eosinophils, 10%; lymphocytes, 3%) (C) MNC of PB, and (D) BM cells. Cells were stained with EO-1 and were assessed by indirect IF with flow cytometry. The dotted line in A to D represents the fluorescence profile of cells stained with control ascites. (A) Ninety-one percent of the cells were positive with EO-1. (B) Thirteen percent of the cells were positive with EO-1.

with EO-1 by the immune rosette test. This was used rather than the cell sorter technique since it permitted the morphologic study of positive and negative cells by preparing and staining smears of the rosetting mixture. Examination of such smears showed that all rosetting cells were eosinophils (Fig 2a) and that a mean of 47.5% of the total number of eosinophils was positive (Table 1). No neutrophils formed rosettes. Eosinophils were also purified with a Percoll gradient from one CML patient's PB since the number of eosinophils is relatively high in this disease. Eighty-eight percent of these eosinophils formed rosettes with EO-1. Moreover, when such rosetting mixtures were sedimented over a Percoll gradient (77%), nonrosette cells were seen to be composed exclusively of neutrophils (Fig 2b). The contaminating eosinophils present in this fraction were also rosette-positive. Most rosette-positive cells recovered at the bottom of the Percoll gradient after lysis of red blood cells were eosinophils (Fig 2c).

To test the reactivity of EO-1 with basophils, relatively enriched preparations (1% to 2% basophils) were obtained by Percoll fractionation of normal PB. We found that most basophils formed immune rosettes with EO-1 (Table 1, Fig 2d). Purified platelets reacted positively with EO-1 in all cases, but the monoclonal antibody used for the control studies did not form any rosettes.

EO-1 antibody was tested against normal (three samples) and CML (two samples) human BM cells separated on F/M gradient. About 5% of the nucleated cells were EO-1–positive in both the normal and CML samples. Of these positive cells, the majority were mature or immature eosinophils (Fig 2e). The remaining positive cells were basophils and a few small lymphoplasmacytoid cells. Neutrophils, myelocytes, and promyelocytes did not react with EO-1. Eosinophils were further purified from the BM of the two CML cases by Percoll sedimentation. The percentages of eosinophils forming rosettes in the two samples were 98% and 53%, respectively (Table 1).

Expression of EO-1 antigen on normal peripheral MNC. A small but regularly detectable percentage of EO-1–positive cells is present in normal MNC (Table 2). We attemped to determine the nature of these cells by fractionating MNC into various subsets and studying their reactivity

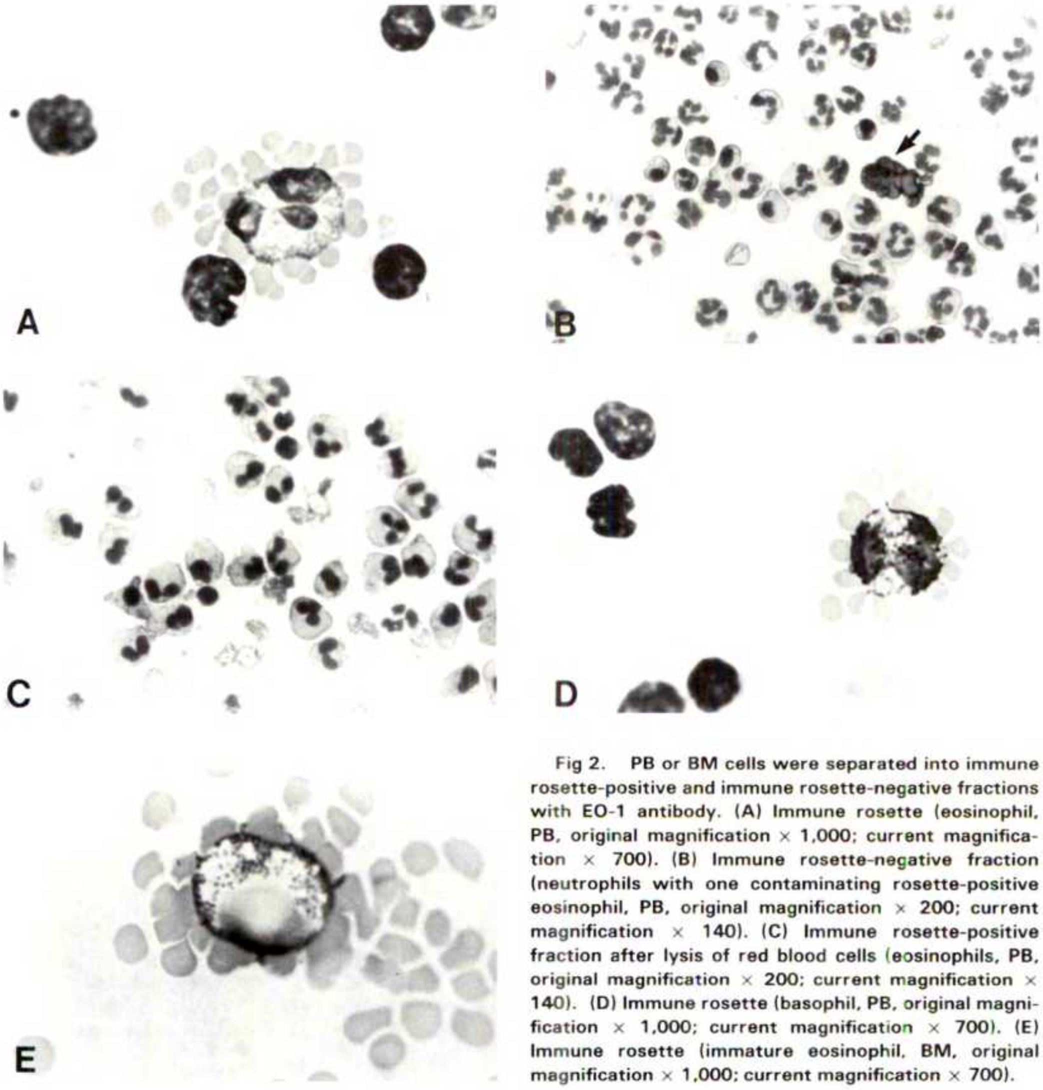

Fig 2. PB or BM cells were separated into immune rosette-positive and immune rosette-negative fractions with EO-1 antibody. (A) Immune rosette (eosinophil, PB, original magnification × 1,000; current magnification × 700). (B) Immune rosette-negative fraction (neutrophils with one contaminating rosette-positive eosinophil, PB, original magnification × 200; current magnification × 140). (C) Immune rosette-positive fraction after lysis of red blood cells (eosinophils, PB, original magnification × 200; current magnification × 140). (D) Immune rosette (basophil, PB, original magnification × 1,000; current magnification × 700). (E) Immune rosette (immature eosinophil, BM, original magnification × 1,000; current magnification × 700).

Table 1. Reactivity of EO-1 With PB and BM Granulocytes and Platelets

Cell Population (No. of Cases)	Percentage of Rosette-Positive Cells*
Eosinophils from normal donors (8)	47.5 ± 28
Neutrophils from normal donors (9)	0
Basophils from normal donors (4)	92.0 ± 9.0
Platelets from normal donors (10)	100†
Eosinophils in CML PB (1)	88
Eosinophils in CML BM (2)	53, 98

*Data are expressed as the mean ± SD of the percentage.

†Reactivity assessed by indirect IF and the immune rosette method.

Table 2. Reactivity of EO-1 With Normal PB MNC

Cell Fraction (No. of Cases)	Percentage of Positive Cells*
MNC (8)	2.0 ± 1.7
T cell fraction (7)†	1.7 ± 0.6
T cell-depleted fraction (7)‡	4.6 ± 2.2
Adherent cells (6)§	5.1 ± 2.5
Null cells (3)‖	6.3 ± 6.5
B cells (1)¶	4.7
B cells (spleen) (1)#	13.0

*Data are expressed as the mean percentage of positive cells with SD, measured by indirect IF.

†T3, 85% ± 5%.

‡T3, 9% ± 2%; Ia, 50% ± 14%.

§Mo2, 85% ± 5%.

‖HNK-1, 35% ± 7%; SmIg, 13% ± 6%.

¶HNK-1, 5%; SmIg, 84%.

#B1, 95.6%.

with EO-1 by indirect immunofluorescence staining (IF). As can be seen in Table 2, separation of the cells into E rosette-positive (E+) and -negative (E−) fractions did not lead to any clear distribution of the EO-1 cells even though most of the EO-1-positive cells seemed to remain in the E− fraction. Subsequent purification of E− cells into null cells and B cell fractions by selectively removing cells forming immune rosettes with erythrocytes coated with monoclonal antibodies (OKT3, B1, and Mo2 for null cell isolation; OKT3, HNK-1, and Mo2 for B cell isolation) by density centrifugation showed that some EO-1-positive cells were present in both fractions (Table 2). In one instance, we purified B cells from the surgically removed spleen of a patient with idiopathic thrombocytopenic purpura (ITP). The percentage of B1$^+$ cells increased from 23% to 95% after the purification procedure, whereas the number of EO-1-positive cells rose from 4% to 13%. It would thus seem that some B cells do react with EO-1. Most of the normal adherent cells (monocytes) were negative.

Reactivity of EO-1 with human cultured cell line. The reactivity of EO-1 antigen was tested against a panel of cultured human cell lines, and the results obtained from indirect IF are shown in Table 3. The strongest reaction for both the intensity of fluorescence and percentage of positive cells was found with pre-B cell lines (NALM-1, NALM-6) and null cell lines. Three out of seven B cell lines also reacted with EO-1. The percentage of positive cells, however, was smaller and the intensity of fluorescence weaker than for the pre-B cell lines. Two erythroleukemia lines (HEL, K562) and one monoblastic line (THP-1) also reacted with EO-1. In contrast, typical myeloid lines such as HL-60, ML-1, ML-2, and KG-1 were all negative. One monocytic line (U937) and all T cell lines were also unreactive.

Expression of EO-1 in hematopoietic malignancies. The reactivity of EO-1 was studied on different types of leukemic cells. Even though the number of cases studied was small, the results (Table 4) were in agreement with those found on cultured cell lines. All cases of null cell ALL were positive. Most B CLL reacted with EO-1 as well as one case of Burkitt's lymphoma. Three samples of CML-BC were also tested. Two were of the myeloid type and were negative with EO-1. The third sample, which was of the lymphoid type and expressed the common ALL antigen (CALLA), reacted with EO-1. T cell malignancies and AML cases were all negative. One case of basophilic leukemia was highly positive.

Biochemical characterization of the EO-1 antigen. Cell surface antigens of intact platelets were radiolabeled with ^{125}I by the lactoperoxidase technique and detergent solubilized. The results of immunoprecipitation with EO-1 and SDS-PAGE are shown in Fig 3. EO-1 recognized an antigen with an apparent MW of 23 kilodalton (kD) under reducing (Fig 3, lane b) and nonreducing (Fig 3, lane e) conditions.

DISCUSSION

In the present report, we describe the production and characterization of a murine monoclonal antibody, EO-1, raised against peripheral eosinophils from a patient with eosinophilic leukemia. A large number of antibody-producing clones was obtained after fusion, but most of the antibodies that reacted with eosinophils were also positive with neutrophils and/or an autologous B cell line. EO-1 was selected because in the first screening it reacted only with the immunizing cells and mainly eosinophils in PB. In addition it

Table 3. Reactivity of EO-1 With Human Cell Lines

Positive Cell Lines*				Negative Cell Lines	
NALM-1 (PrB)	100	HEL (M)	32	RPMI 8866 (B)	U937 (M)
NALM-6 (PrB)	100	K562 (M)	3	DAUDI (B)	JM (T)
NALM-16 (N)	23	THP-1 (M)	66	RAJI (B)	MOLT4F (T)
REH (N)	15			P3HR1 (B)	HSB (T)
SERAFINA (B)	21			HL-60 (M)	CEM (T)
RAEL (B)	9			ML-1 (M)	
ARH77 (B)	5			ML-2 (M)	

Abbreviations: PrB, pre-B cell line; N, null cell line; B, B cell line; M, myeloid-monocytoid cell line; T, T cell line.

*Data are expressed as the percentage of positive cells.

Table 4. Reactivity of EO-1 With Fresh Leukemic Cells

Classification	No. Positive/No. Tested
Null ALL (CALLA$^+$)	5/5
B CLL (B1$^+$)	5/7
Burkitt's lymphoma (B1$^+$)	1/1
CML-BC lymphoid (CALLA$^+$)	1/1
Myeloid	0/2
AML	0/4
T ALL	0/1
T CLL	0/1
Basophilic leukemia*	1/1

*T3-, Mo1-, B1-, IF10-, J5-negative cells.

was observed that in the BM preparations, EO-1 was negative with mature and immature neutrophils (myelocytes, promyelocytes) and positive with mature and immature eosinophils, basophils, platelets, and some lymphoid cells. It remains to be seen, however, whether all eosinophils express the EO-1 antigen or whether there exist distinct subsets of eosinophils in terms of the EO-1 expression. The selective reactivity of EO-1 with eosinophils is interesting since it is, to our knowledge, the first monoclonal antibody that can distinguish eosinophils from neutrophils and immature myeloid cells. This restricted specificity may be useful for the analysis of differentiation and function of eosinophils.

During these studies on normal blood, we noticed that a small percentage of positive MNC was frequently found. The exact number of positive cells was difficult to assess since platelets, which are present in these preparations, induce a high background and their adherence to monocytes can artificially increase the percentage of apparently reactive cells.[16] However, we tried to determine the nature of these EO-1–positive cells by fractionating MNC preparations into various subsets. No clear distribution of these cells could be obtained because a few EO-1–reactive cells were found in every fraction (E+, E−, null, B, adherent cells). One B cell preparation, however, purified from spleen cells from ITP, contained 13% EO-1–positive cells in a 95% B1–positive cell fraction. These data together with the positive plasmacytoid cells found in the BM samples seem to indicate that some B lineage cells express the EO-1 antigen.

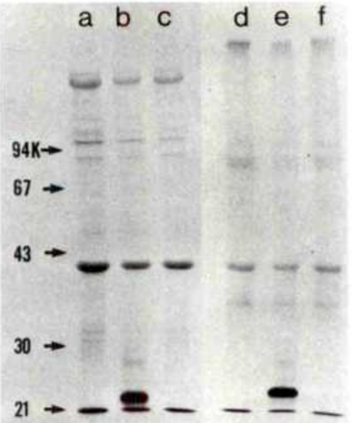

Fig 3. Immunoprecipitation of EO-1 antigen. Platelet membranes were labeled and extracted as described in Materials and Methods. The number shown indicates the mol wt of the protein standards. The EO-1 antigen was analyzed in the absence (lane e) or presence (lane b) of dithiothreitol (DTT). Lanes a and c and lanes d and f show nonspecific bands obtained when unrelated IgGI antibodies were used for control. EO-1 recognized the 23-kD component when electrophoresis was performed under reducing and nonreducing conditions.

Among all the cell lines tested, pre–B cells (NALM-1, NALM-6) and null cells (NALM16, Reh) gave the highest reaction both in intensity and in the reacting number of positive cells. In contrast, typical myeloid lines (HL-60, KG-1, ML-1, ML-2) and all the T cell lines were negative. Similar results were also obtained with fresh malignant cells. Even though the total number of cases available for study was small, we could show that lymphoblastoid malignancies of the null cell type (CALLA$^+$ null cell ALLs) were positive as well as most B cell CLL. These results are interesting because recent data indicate that most null cell ALLs seem to be associated with the B cell lineage.[17,18]

Monoclonal antibodies such as BA-2, DU-ALL-1, and FM8[19–21] recognize pre–B cell–associated antigens and react with B cells, platelets, and polymorphonuclear neutrophils. These antibodies recognize a molecular weight (mol wt) of 24 kilodaltons (kD), which is similar to the mol wt of the EO-1 antigen. However, EO-1 antibody appears to be distinct from these previously described monoclonal antibodies recognizing the 24-kD phenotypic marker of CALLA-positive cells on the basis of specificity. EO-1 does not react with neutrophils. Studies using coprecipitation or antibody blocking will clarify the relationship between the EO-1 antigen and the 24-kD antigen recognized by these monoclonal antibodies.

The reactivity of EO-1 antibody with both eosinophils and basophils is not surprising since both cell types are known to share the same properties, and it has been suggested that they derive from a common progenitor.[22–24] Antibodies recognizing surface determinants shared by platelets and granulocytes, as is the case for EO-1, have also been reported (B37.3, A4).[7,25] The reactivity of EO-1 with B lineage cells was more unexpected. However, there are already numerous examples of unrelated hematopoietic cells bearing similar surface markers. The CALLA antigens, for example, are present on pre–B cells and neutrophils.

Basophilic granulocytes are known to express Fc_ϵ receptors,[26] and more recent reports have established the existence of Fc_ϵ receptors on eosinophils[27] and platelets.[28,29] This cellular distribution of Fc_ϵ receptors seems to correlate with that found for EO-1. However, the evidence that EO-1 does not react with RPMI 8866 (B lymphoblastoid line) and U937, both of which have high levels of Fc_ϵ receptors, and that the inhibition by EO-1 antibody against IgE-rosette binding to eosinophils was not seen (Professor M. Capron, Pasteur Institute, Lille, France, personal communication March 1985) shows that EO-1 does not react with Fc_ϵ receptors, or at least the binding site of the receptor. More experiments will be needed to characterize the relationship between EO-1 and the Fc_ϵ receptors of eosinophils.

In conclusion, we report that murine IgGl monoclonal antibody (EO-1) was produced from mice immunized with purified eosinophils from a patient with eosinophilic leukemia. This antibody precipitated a 23 kD mol wt component. The EO-1 antibody reacted with mature and immature eosinophils, basophils, platelets, and a few lymphoid cells, whereas it did not react with neutrophils and immature myeloid cells. This antibody may be useful for the analysis, isolation, and study of eosinophils and basophils.

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A Monoclonal Antibody Against an Erythrocyte Ontogenic Antigen Identifies Fetal and Adult Erythroid Progenitors

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A murine monoclonal antibody (MoAb) designated FA6-152 has been obtained by immunizing mice with fetal erythrocytes. This antibody agglutinates fetal but not adult erythrocytes. Among blood cells, this antibody bound to both adult and fetal monocytes, platelets, and reticulocytes, but did not react with lymphocytes and granulocytes. Fluorescent labeling of marrow cells and of in vitro BFU-E, CFU-GM, and CFU-MK-derived colonies has shown that the antigen defined by FA6-152 MoAb was absent from the granulocytic precursors and was detected on the megakaryocytic lineage at a later stage of differentiation than the platelet-specific markers. In contrast, the antigen appeared as a very early marker of the erythroid differentiation since all erythroblasts, including proerythroblasts, were labeled even before the expression of glycophorin A. Cells from adult marrow and fetal liver were sorted with the FA6-152 MoAb and studied by electron microscopy and cell culture. The negative fraction contained granulocytic, monocytic, and megakaryocytic precursors, whereas the positive fraction was devoid of these precursors and contained monocytes, erythroblasts at all stages of maturation, and a homogenous population of blasts. Cultures have shown that the only hematopoietic progenitors present in this positive fraction were CFU-E and some BFU-E. The antigenic density was related to the differentiation stage of the erythroid progenitors. In conclusion, this antibody is similar to the previously described 5F1 MoAb (Bernstein and Andrews, *J Immunol* 128:876, 1982; and Andrews et al, *Blood* 62:124, 1983) and provides a useful probe for studies leading to improved understanding of normal and malignant erythroid differentiation.

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NUMEROUS ONTOGENIC changes are observed in the biology of the erythrocyte. The most striking ontogenic event concerns hemoglobin switching. Nevertheless, blood group antigens also exhibit numerous important developmental changes. The antigens of the A, B, and H systems are slightly expressed at the third week of the intrautero life.[1] As early as the 40th day of fetal life, the antigens of the ABH, Duffy, JK, and rhesus blood group systems are fully expressed on the surface of the erythrocytes. However, fetal erythrocytes differ from adult erythrocytes by the arborized pattern of the carbohydrate structure of their glycolipids and glycoproteins.[2,3] This difference is now considered to be the basis of i-to-I antigen conversion.[4-6] The i/I expression during adult erythroid differentiation mimics the ontogenic changes. Indeed, i antigen is preferentially detected on immature erythroblasts whereas increasing expression of I antigen is observed during terminal erythroid differentiation.[7,8] However, at the level of adult erythroid progenitors, ie, CFU-E and BFU-E, I antigen predominates over i antigen.[9] Therefore, ontogenic erythrocyte antigens can be helpful markers in studies of cellular differentiation and may be also useful for identification of fetal erythrocytes in maternal blood during pregnancy.

Our purpose was to define new ontogenic erythrocyte antigens with monoclonal antibody (MoAb) obtained by immunizing mice with fetal erythrocytes and to investigate the expression of such antigens during the erythroid differentiation.

MATERIALS AND METHODS

Immunization protocol. Balb/c mice (IFFA Credo, Lyon, France) were immunized using a suspension of 20-week-old fetal red cells (RBC). These heparinized RBC were washed three times in saline (0.15 mol/L NaCl), and 20 × 10^6 cells were injected on day 1 and day 18 intraperitoneally. Three days after the last immunization, spleen cells were prepared, and fusion was performed as previously reported.[10,11]

Supernatant screening. Culture supernatants were tested by a hemagglutination test using papain- and bromelain-treated adult and fetal RBC. The subsequent reactions were performed in microtubes by mixing 20 μL of the aforementioned RBC suspension and 20 μL of supernatants. The mixture was incubated for 30 minutes at 37 °C and then read with a microscope for agglutination.

Cloning. The cells secreting an antibody of interest were cloned by the limiting dilution technique. The cells taken from one well were brought into suspension with a feeder layer (thymocytes from Balb/c mice) and distributed in 96-well plates so as to obtain an average cell density of 5, 1, and 0.5 cells per well. Two successive clonings were performed. For further studies, the culture supernatant or ascitic fluid was used as sources for the MoAb.

Characterization. Identification of the MoAb was carried out using a panel of adult different erythrocyte phenotypes; agglutination tests were performed by adding one volume of a 10%-treated RBC suspension to an equal volume of culture supernatant or ascitic fluid. Both macroscopic and microscopic readings were made after one hour of incubation at 22 °C. Immunoglobulin class and subclass of the monclonal antibody were determined by immunodiffusion of ascitic fluid against subclass-specific antiimmunoglobulin antisera (Meloy Laboratories, Springfield, Va).

Samples. Adult RBC of known phenotype or fetal blood samples obtained from therapeutic abortion or fetoscopy for antenatal diagnosis were used. The blood cells were collected into heparin, washed three times in saline (0.15 mol/L NaCl), mixed in a glycerol (17.5%), mannitol (1.44%), and NaCl (0.32%) solution, and frozen in liquid nitrogen. Adult RBC with rare phenotypes were thawed and bromelin treated. Liver and thymus cells were removed one hour after delivery from therapeutic abortion, placed into Dulbecco's medium containing 10% foetal calf serum, and subsequently frozen.•

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Isolation of the cells. Normal mononuclear cells from heparinized peripheral blood or bone marrow were separated by Ficoll-metrizoate gradient density centrifugation (Lymphoprep, Nyegaard, Sweden; d:1077).[12] Adherent and nonadherent peripheral blood mononuclear cells were separated by a two-hour adherence on plastic Petri dishes. The granulocytes from the pellet of the Ficoll-metrizoate gradient density centrifugation were separated from RBC by dextran sedimentation. Platelets were obtained from platelet-rich plasma. Reticulocytes were separated on a phthalate oil density gradient[13] from a patient with autoimmune hemolytic anemia.

Bone marrow cells were isolated from aspirates obtained from donors for bone marrow transplantation procedures. Nucleated cells were either obtained from a buffy coat preparation or after Ficoll-metrizoate gradient density centrifugation. Blood cells from 40 patients with acute leukemia were also tested.

Cell lines. Five lymphoblastoid cell lines, five Burkitt cell lines (including Daudi and P3HR1), three T cell lines (Jurkatt, Molt 4, HSB 2), HL 60 (a promyelocytic cell line), U 937 (a monoblastic cell line), two erythroleukemic cell lines (HEL and K 562), and two hybrid cell lines[14] between K 562 and P3HR1 and K 562 and DAUDI (Putko and Dutko, respectively) were investigated. In addition, HL 60 and U 937 were studied before and after 12-0-tetradecanoyl phorbol-13-acetate (TPA) and 1.23 dihydroxy-vitamin D_3 induction. All cell lines were cultured in RPMI medium (Boehringer, Mannheim, West Germany) with 10% fetal calf serum (Boehringer).

Fluorescent labeling on separated cells. Supernatant or ascitic fluid was used undiluted or 100-fold diluted in sterile phosphate-buffered saline (PBS), respectively. Unfixed cells were incubated at 4 °C for 30 minutes with the antibody. After three washes in cold PBS, cells were incubated with a 10^{-2}-diluted fluorescein-conjugated goat $F(ab')_2$ fragment directed against mouse IgGs (Cappel Laboratories, Cochranville, Pa) for 30 additional minutes, washed, smeared, and fixed for one minute with pure methanol. They were examined under a Zeiss episcope equipped with appropriate filters for fluorescein and rhodamine and for phase contrast.

Cell sorting. Fluorocytometric analysis and cell sorting were performed with an Ortho 50 H cytofluorograf (Ortho Diagnostics, Westwood, Mass) as previously described.[9] Light-density marrow cells from bone marrow transplantation donors were frozen, thawed just before the cell sorting experiment, labeled with an antibody by an indirect fluorescent technique (see the previous section), and kept in ice until cell sorting. Collected cells from the two fractions were used for clonogenic assays, cytocentrifuge preparations, and electron microscopic (EM) studies (see the following section).

The cytocentrifuge slides were stained by the May-Grunwald-Giemsa technique or labeled by an indirect fluorescent technique after methanol fixation with rabbit polyclonal antibodies against myeloperoxidase[15] or von Willebrand Factor (vWF) (Dakopatts, Copenhagen, Denmark). In one experiment, adult and fetal RBC were mixed in two different percentages (10% fetal and 1% fetal) and subsequently sorted. Frankly positive RBC were obtained and allowed to settle onto polylysine-coated slides.[16] Fluorescent labeling with a rabbit antihuman γ-globin chain[17] was subsequently performed after ethanol–glacial acetic acid fixation.

Clonogeneic assays. The clonogeneic assays for BFU-E, CFU-E, CFU-GM, and CFU-MK were performed by the plasma clot technique.[18] The stimulating factors were either phytohemagglutinin leukocyte-conditioned medium (PHA-LCM) for CFU-MK and CFU-GM colony growth and PHA-LCM plus 1 IU/mL porcine erythropoietin (Epo) (CNTS, Paris) for CFU-E and BFU-E. These cultures were performed with two goals in mind.

First, the cultures allow the preparation of enriched populations of erythroblasts, granulocytes-monocytes, and megakaryocytes from BFU-E, CFU-GM, and CFU-MK cultures, respectively. Studies at day 6 or 7 of the cultures permit the characterization of populations of immature erythroblasts, granulocytes, or megakaryocytes, whereas similar studies at day 13 permit the investigation of mature cells. Fluorescent labeling with FA6-152 MoAb was performed directly on the Petri dish as previously described.[19]

Second, the cultures have permitted us to study the types of progenitors contained in the two fractions collected with the cell sorter. All cultures were performed in the presence of PHA-LCM and Epo. Cultures were studied at day 7 for CFU-E, at day 12 for CFU-GM, mature BFU-E, and CFU-MK, and at day 16 for primitive BFU-E. They were investigated by fluorescent labeling with three MoAbs, ie, LICR LON R10 (anti-glycophorin A),[22] C 17 (antiplatelet glycoprotein IIIa),[21] and 80 H 5 (anti-SSEA-1 antigen),[22] specific for the erythroid, megakaryocytic, and granulocytic-monocytic lineages, respectively. Each dish was entirely scanned.

Ultrastructural studies. The two fractions of cells (positive and negative) collected with the cell sorter were fixed by 1.25% glutaraldehyde in Gey's basic salts[23] for ten minutes, washed and incubated in 2 mg/mL diaminobenzidine medium (Sigma Chemical Co, St Louis).[24] The cells were then washed, postfixed, dehydrated, and embedded in epon. Thin sections were examined with a Philips electron microscope. This technique permits monitoring of different peroxidase activities, ie, myeloperoxidase, platelet peroxidase, and the peroxidase activity of hemoglobin.[25] Cells were also studied by the immunogold technique with the FA6-152 MoAb.[25]

RESULTS

Reactivity with erythrocytes. Two weeks after fusion, 320 wells out of 1,024 contained hybrid cells. Twelve were secreting antibodies against adult and fetal human RBC. Only one well, ie, FA6-152, produced an antibody that agglutinated only fetal RBC. The supernatant was tested against a panel of 69 adult RBC typed in the ABO, rhesus, Kell, Duffy, Kidd, P, MNSs, Lutheran, Colton, Cartwright, Xg, Sid, and Dombrock systems, a panel of 16, 18, 20, 22, 24, 27, 30, and 32-week-old fetal RBC and a panel of 23 cord blood RBC. Only fetal RBC up to 32 weeks old were agglutinated by the FA6-152 MoAb (Table 1). This antibody was characterized as an IgG1 antibody. Even adult RBC with a rare phenotype such as TJa-, Cartwright –, Gerbich –, Vel –, Colton –, kpb –, U –, i, PK, Bombay, and Celano were not agglutinated by the MoAb.

By fluorescent technique, adult erythrocytes were unlabeled, but some adult reticulocytes were faintly stained. About 50% of fetal RBC were labeled, whereas 10% to 20% cord blood RBC were stained. Cell sorting of artificial mixtures of fetal and adult erythrocytes was performed. For an initial mixture of 10% fetal RBC, 95% fetal RBC (labeled by an anti–γ-globin chain) were present in the positive fraction whereas for a mixture containing 1% fetal RBC, 60% fetal RBC were present in the positive fraction.

Reactivity with other blood cells. In the adult, 30% to 40% of the blood mononuclear cells were labeled. These cells were larger than the others and exhibited some granulation in phase-contrast microscopy. More than 90% of the adherent cells were stained, whereas less than 1% were labeled in the nonadherent population. Granulocytes were unlabeled. All the platelets from several donors were labeled by the antibody in a double fluorescent procedure using a polyclonal

Table 1. Reactivity of FA6-152 With Erythrocytes

Adult RBC	No.	Agglutinated	Cord RBC	No.	Agglutinated	Fetal RBC (Age)	No.	Agglutinated
A_1	10	—	A_1	15	—	A (18 wk)	4	++
A_2	5	—	A_2	5	—	O (20 wk)	5	++
A_3	3	—	B	6	—	O (22 wk)	7	++
A_x	3	—	O	8	—	A (24 wk)	5	++
B	7	—				O (27 wk)	4	++
O	41	—				O (30 wk)	4	++
						O (32 wk)	3	++

antifactor vWF to identify the platelets. EM studies by the immunogold technique have clearly confirmed that all platelets and monocytes bind the antibody whereas lymphocytes and granulocytes were devoid of the antigen.

Reactivity with hematopoietic precursors—Labeling of blood cell precursors. Ten percent to 35% of the marrow cells were labeled by the antibody. When examined by phase contrast, they consisted of erythroblasts at all stages of maturation, megakaryocytes, and monocytes. Granulocytic precursors were unlabeled. Similar results were obtained at the EM level. To determine more precisely the stage of maturation of the cells labeled by FA6-152, fluorescent labeling was performed on BFU-E–, CFU-GM–, and CFU-MK–derived colonies. All the erythroblasts composing a BFU-E colony were stained by the antibody at the early or late days of culture. Blood BFU-E colonies were stained as early as day 5 of culture. In similar experiments, MoAb directed against glycophorin A labeled the BFU-E–derived colonies, but not before day 8 or 9 of culture. Pure granulocytic colonies remained entirely unlabeled by the MoAb. Only large macrophages were intensively stained in the CFU-GM assay. Large mature megakaryocytes shedding platelets were intensively stained by the FA6-152 MoAb. At day 7 of culture when a large number of immature megakaryocytes were present, only 60% of the megakaryocyte labeled by antifactor vWF were reactive with FA6-152 MoAb. In particular, small megakaryocyte precursors were weakly labeled or unstained. Results were similar in colonies cultured from fetal progenitors, but with a more intense staining on fetal erythroblasts and megakaryocytes than on their adult counterparts.

Table 2. Reactivity of FA6-152 With Several Hematopoietic Cell Lines

	Percentage
Lymphoblastoid (five cell lines)	—
Burkitt's lymphoma (five cell lines)	—
T-ALL (three cell lines)	—
U937	3.5 ± 2
TPA induced	2 ± 1
Dehydroxy-vitamin D_3	3.5 ± 2
HL 60	—
TPA induced	—
HEL	75 ± 10
K 562	2 ± 2
Putko (hybrid between K 562 and P3 HR-1)	>90
Dutko (hybrid between K 562 and Daudi)	>90

Abbreviation: T-ALL, T cell acute lymphocytic leukemia.

Hematopoietic cell lines and leukemic cell. Among the cell lines tested, only HEL, Putko, and Dutko expressed the antigen defined by FA6-152 on a majority of cells. This antigen was also detected on a minority of K 562 and U 937 cells. (Table 2) Fifteen cases of acute lymphoblastic leukemia were investigated, and all cells were negative. In 25 cases of acute myeloid leukemia, only the cases containing a mature or immature monocytic component (M_4 and M_5) according to the French-American-British (FAB) classification[26] were stained; M_1, M_2, and M_3 cases were always negative.

Morphologic features of the marrow cell fraction collected by cell sorting. These experiments were performed on bone marrow aspirates obtained from normal donors for bone marrow transplantation. These samples were heavily

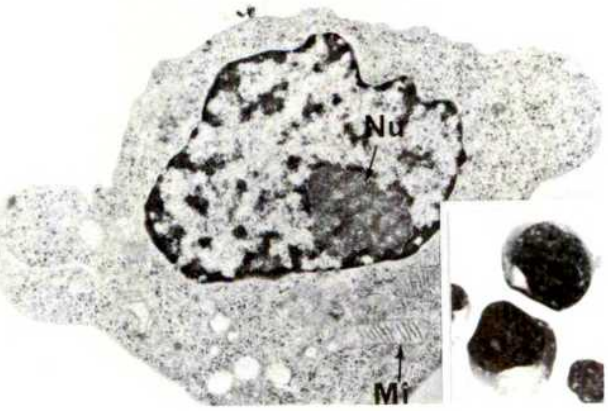

Fig 1. Blast cells present in the positive fraction. EM examination of a blast cell present in the positive fraction. EM shows that this cell cannot be identified since no peroxidase activity can be detected. The nucleus contains mainly euchromatin: the heterochromatin is present along the nuclear membrane and surrounds the nucleolus (Nu). The cell membrane is smooth, without pseudopods, but two symetric cytoplasmic blebs are seen. The cytoplasm contains rare cisternae of endoplasmic reticulum but numerous polysomes: the mitochrondria (Mi) are large and the Golgi apparatus exhibits a number of vesicles. Several vacuoles are present, and an annulate membrane can be observed bottom right. Note the absence of rhopheocytosis and ferritin molecules (original magnification × 5,200; current magnification × 4,472). (Inset) May-Grunwald-Giemsa staining of two blast cells present in the positive fraction. The blast cells are large with a basophilic cytoplasm. The nucleus is slightly indented and contains a large nucleolus.

Table 3. Progenitor Cell Growth From the Two Fractions Collected by Cell Sorting From Adult Bone Marrow

Experiment No.	CF	Percentage of CF	CFU-E		Day 12 BFU-E		Day 16 BFU-E		CFU-GM		CFU-MK	
			per 10^5 CF	Percentage*	per 10^5 CF	Percentage*	per 10^5 CF	Percentage*	per 10^5 CF	Percentage*	per 10^5 CF	Percentage*
1	Positive I	26	275 ± 24	52.5	8 ± 4	2	ND		4 ± 4	1.1	0	0
	Negative	65	115 ± 11	37	134 ± 23	89.5	ND		126 ± 42	91	26 ± 5	93
2	Positive I	34	235 ± 33	53.5	3 ± 2	1.1	3 ± 2	2.5	29 ± 15	4.8	0	0
	Negative	63	79 ± 10	33	148 ± 35	93	59 ± 9	92.5	308 ± 62	93	24 ± 8	80

Abbreviations: CF, cell fraction; ND, not determined.

*The percentage is calculated as follows: CFU/10^5 sorted cells × percentage of viable cells in fraction/(CFU/10^5 unsorted cells). In both cases, bone marrow from a marrow donor for marrow transplantation was separated by Ficoll density centrifugation, stored frozen with 10% dimethylsulfoxide in liquid nitrogen, and thawed for sorting.

The positive fraction contained only bright fluorescent cells, whereas the negative fraction contained the clear negative fraction and the cells collected in the shoulder of fluorescence (Fig 2).

contaminated by cells of blood origin, thus explaining the high percentage of lymphocytes and monocytes.

Thirty percent to 40% of the examined cells were labeled. This positive fraction comprised 79% to 85% monocytes; 1% to 2% additional immature monocytes were also present. The positive fraction included all the erythroblasts and proerythroblasts. No identifiable myeloid precursors were present. This fraction also included 3% to 8% blast cells that were not labeled by polyclonal antibodies against factor vWF or myeloperoxidase. These cells contained carbonic anhydrase I, another early erythroid marker.[27]

The two fractions collected were subsequently examined by EM. The negative fraction contained all the neutrophil, eosinophil, and basophil precursors. Promonocytes synthesizing myeloperoxidase (ie, presence of a peroxidase activity in the Golgi apparatus and the endoplasmic reticulum) or having terminated this synthesis and rare promegakaryoblasts identified by the typical platelet peroxidase were also present. In the positive fraction, the majority of cells examined were monocytes that were rather mature since no synthesis of myeloperoxidase was detected. Proerythroblasts were clearly identified by the presence of ferritin in rhopheocytosis vesicles and in small clusters in the cytoplasm. A homogenous population of blast cells without any cytochemical or morphologic criteria for identification were present (Fig 1). They differed from the blast cells of the negative fraction by their large size, abundant cytoplasm rich in free ribosomes, mitochondria of large size, and granules located in the Golgi apparatus zone. The slightly indented nucleus contained a large and multiple nucleolus.

Hematopoietic progenitor content of the adult bone marrow cell fractions collected by cell sorting. After indirect fluorescent labeling with FA6-152 MoAb, marrow cells were analyzed and sorted on the basis of fluorescent intensity (Tables 3 and 4). A typical histogram is shown in Fig 2. It was characterized by a clear population of negative cells and a marked shoulder preceding the FA6-152 bright cells. We could determine that this shoulder was contaminated by 2% to 10% negative cells when control IgG1-labeled cells were studied. In contrast, the FA6-152 bright cells contained less than 0.05% negative cells.

In two experiments, the positive fraction (positive I) only contained the FA6-152 bright cells, whereas the negative fraction also included the shoulder of fluorescence (Fig 2A). Results are shown in Table 3. More than 50% of the CFU-Es were grown from the positive I fraction, largely in the absence of all the other hematopoietic progenitors. The CFU-Es giving rise to eight to 32 cell colonies were predominantly found in this fraction, whereas most of the CFU-Es giving rise to 32 or more cells were detected in the negative fraction containing both truly negative cells and cells expressing a low antigenic density of the antigen (Fig 3).

In two additional experiments (Table 4), FA6-152 bright cells and a part (experiment 3) or all (experiment 4) of the cells in the shoulder of fluorescence and therefore corresponding to cells exhibiting a low antigenic density were included in the positive fraction (positive II fraction). Most or all CFU-Es and a part of the day 12 BFU-E were grown from the positive II fraction, whereas all the other hematopoietic progenitors including the day 16 BFU-E were absent from this fraction. The positive sorting of cells expressing a very low antigenic density in only experiment 4 may explain the differences between experiments 3 and 4.

Finally, cells with a high antigenic density (positive I) were separated from the cells with a low antigenic density (intermediate) (Table 4, experiment 5, Fig 2B). The positive I fraction contained only a part of the CFU-Es, whereas the intermediate fraction included the other CFU-Es and a

Table 4. Progenitor Cell Growth From the Two Fractions Collected by Cell Sorting From Adult Bone Marrow

Experiment No.	CF	Percentage of CF	CFU-E		Day 12 BFU-E		Day 16 BFU-E		CFU-GM		CFU-MK	
			per 10^5 CF	Percentage*	per 10^5 CF	Percentage*	per 10^5 CF	Percentage*	per 10^5 CF	Percentage*	per 10^5 CF	Percentage*
3	Positive II	34	402 ± 43	89	74 ± 12	29.8	0	0	91 ± 18	10.5	ND	
	Negative	63	24 ± 6	5.3	86 ± 23	64.3	73 ± 16	96	409 ± 41	87.4	ND	
4	Positive II	40	480 ± 54	95	305 ± 46	71.5	ND		48 ± 8	5.9	ND	
	Negative	60	0	0	68 ± 14	23.4	ND		469 ± 52	87.8	ND	
5	Positive I	34	235 ± 33	53.5	3 ± 2	1	3 ± 2	4.6	29 ± 15	8	0	0
	Intermediate	5	1,100 ± 185	36.3	1,140 ± 263	69.7	60 ± 32	7.4	40 ± 15	1	0	0

*The percentage was calculated as in Table 3.

In contrast to Table 3, the positive fraction also contained a part of the shoulder of the fluorescent histogram (experiment 3), or the whole shoulder (Experiment 4). In experiment 5, the intermediate *fraction only contained the shoulder, whereas the positive fraction (see experiment 2) only contained the FA6-152 bright cells.

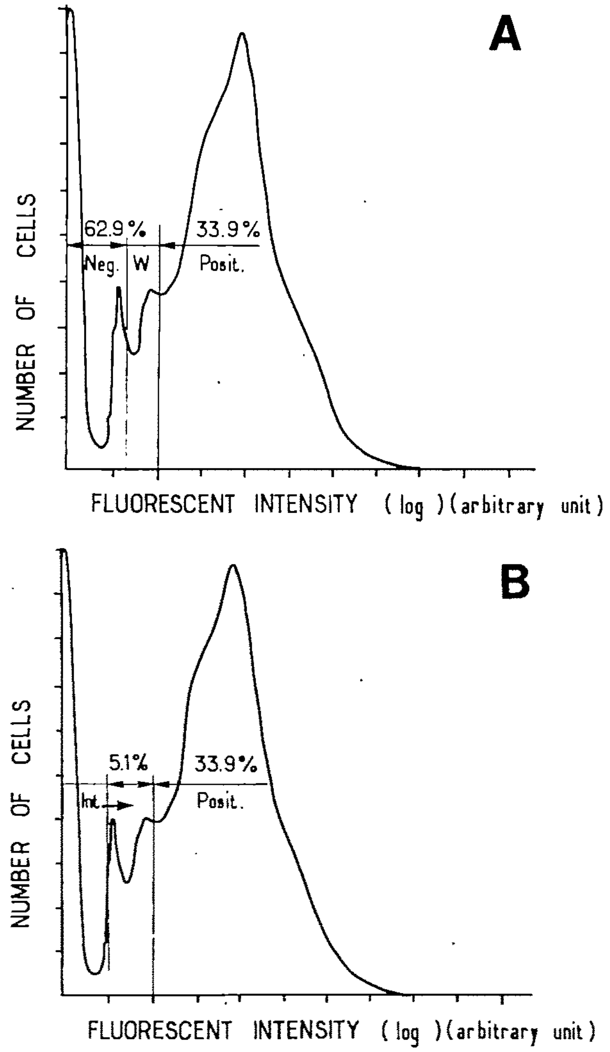

Fig 2. Fluorescence histograms of the bone marrow cells labeled with FA6-152 MoAb. The histogram was characterized by clearly negative and positive cells. However, a marked shoulder in the curve was constantly observed. (A) In experiments 1 and 2, the negative fraction contained a part of the cells located at the shoulder of the fluorescent intensity. The positive fraction contained only bright fluorescent cells. A window of cells between the two fractions was discarded. (B) In experiment 5 (identical bone marrow cells as in experiment 2), the positive fraction was collected as in (A). The other fraction, called intermediate, contained all the cells located at the shoulder of the fluorescence curve.

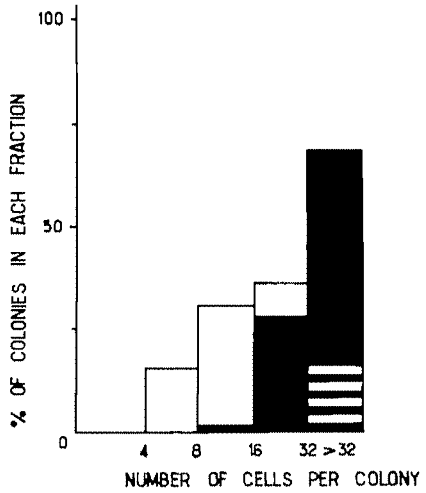

Fig 3. Size of the day 7 erythroid colonies present in the positive and negative fraction separated as in Fig 1A. This figure illustrates the size of the colonies in the positive and negative fraction. The colonies were classified in four classes. The four- to eight-cell colonies were not included in the quantitation of CFU-Es (Tables 2 to 6).

part of day 12 BFU-Es. The other hematopoietic progenitors were absent from these two fractions.

To demonstrate that the absence of BFU-E and CFU-GM in the positive fraction collected was not the consequence of the high concentration of monocytes as previously described,[28] we studied the positive fraction after adherence (Table 5). No significant differences in the number of colonies were observed. In addition, the positive and negative fractions were remixed after sorting; the number of CFU-E and CFU-GM observed did not markedly differ from the sum of the number of colonies found in the two fractions, thereby indicating that no inhibitory cells were present in the two fractions.

Hematopoietic progenitor content of the fetal liver cell fractions collected by cell sorting. Concerning experiments in Table 1 on the adult marrow, we collected the bright stained cells as the positive fraction and the other as the negative (Table 6). The positive fraction contained more than 90% erythroblasts, 72.5% of the CFU-E, and 34% of the mature BFU-E; in contrast, the negative fraction contained most if not all the primitive BFU-E, CFU-GM, and CFU-MK.

DISCUSSION

In this report, we have described a MoAb obtained by immunizing mice with human fetal erythrocytes. The purpose of this immunization was to detect ontogenic antigens present on fetal erythrocytes but not on adult erythrocytes. The antigen detected by FA6-152 MoAb has this characteristic. Therefore, we have extensively studied the expression of this antigen on adult and fetal hematopoietic cells including

Table 5. Adherence and Mixing Experiments

	CFU-E	CFU-GM	BFU-E
Positive fraction			
Before adherence	75.5 ± 5	10 ± 2	2 ± 1
After adherence	65 ± 8	8 ± 5	3 ± 2
Negative fraction	41.5 ± 10	159 ± 22	
Positive fraction (before adherence) + negative fraction	140 ± 25	177 ± 25	

Cells from experiment 2 were grown in triplicate at 3 × 10^4 cells per mL for each fraction. The positive fraction was studied before and after one-hour adherence to plastic. A total of 3 × 10^4 positive and 3 × 10^4 negative cells were also mixed.

Table 6. Cell Sorting of Fetal Liver Cells

	Day 5 CFU-E		Day 8 BFU-E		Day 12 BFU-E		Day 12 CFU-GM		Day 12 CFU-MK	
Percentage	per 10^5 CF	Percentage	per 10^5 CF	Percentage	per 10^5 CF	Percentage	per 10^5 CF	Percentage	per 10^5 CF	Percentage
Positive fraction 60%	372 ± 44	40.7	212 ± 28	32.26	182 ± 24	7.8	15 ± 4	4.6	0	0
Negative fraction (40%)	210 ± 18	26.7	620 ± 75	62.8	2,933 ± 340	83.8	303 ± 27	93.2	62.5 ± 24	78.1

Liver cells from a 20-week-old fetus were frozen, then thawed for sorting experiments. Cells were labeled by FA6-152 and then separated as in Fig. 2A in two fractions and cultured. Cultures were performed in triplicate at 3 × 10^4 cells per mL. Results are expressed per 10^5 cells in each fraction.

progenitors and on continuous cell lines. On adult peripheral blood, this antigen was not detected on erythrocytes. Some reticulocytes, most if not all monocytes, and all platelets bore the antigenic determinant defined by FA6-152. It was not detected on granulocytes and lymphocytes. A similar cellular reactivity was found in four (12- to 20-week-old) fetuses with the exception of a reactivity on erythrocytes. Agglutination of neonatal RBC was not observed, whereas fluorescent labeling was detected in 10% to 20% of the neonatal RBC. In addition, the antigen defined by FA6-152 is not specific for the hematopoietic tissue since it was detected on frozen sections from several adult or fetal organs in the perivascular spaces of the vessels. Reactivity was therefore detected in the small intestine, kidney, liver, and thyroid.

The antigen could not be biochemically characterized by immunoprecipitation and Western blotting on SDS-PAGE (Cartron, Centre National de Transfusion, Paris, 1985). The absence of destruction of the antigen by several proteolytic enzymes such as trypsin and pronase suggests that this antigen is a glycolipid or a carbohydrate structure associated with a glycolipid. MoAbs against the carbohydrate structure have permitted a characterization of such ontogenic ubiquitous antigens as the SSEA-1 antigen, which are also differentiation markers.[29]

Bone marrow, fetal liver hematopoietic precursors and also their counterparts obtained in vitro from BFU-E, CFU-GM, and CFU-MK differentiation were investigated. In the adult as in the fetus, FA6-152 MoAb labeled all erythroblasts including normoblasts. We could demonstrate that the antigen identified by the FA6-152 MoAb was synthesized at an earlier stage of erythroid differentiation than glycophorin A since the colonies derived from blood BFU-E were labeled as early as day 5, whereas a MoAb against glycophorin A did not stain the colonies before day 8. In contrast, the present antigen was expressed in megakaryocytic differentiation later or at the same time as the specific platelet proteins.[19,30] In addition, investigation by EM of the positive and negative marrow cell fractions obtained by cell sorting as well as unsorted marrow cells has shown that promonocytes were devoid of the antigen identified by the FA6 152-MoAb, thereby indicating that this antigen is also a late marker of the monocyte differentiation. All these results are summarized in Fig 4. Considering the results observed in the adult, the reactivity of FA6-152 is in many ways similar to two previously described MoAbs, ie, 5F1[31] and 20/3.[32] Both of them are IgM MoAb obtained by immunization of mice with acute myelomonocytic leukemic cells or normal peripheral mononuclear cells. The reactivity of 5F1 and 20/3 on fetal cells has not been published. We recently found that 5F1 also binds to some fetal erythrocytes.

We investigated the reactivity of FA6-152 with hematopoietic progenitors. It clearly appeared that FA6-152 stained only the erythroid progenitors. Interestingly, there was a marked increase in the antigenic density from the BFU-E to the CFU-E in the adult as in the fetus. The primitive BFU-E[33] did not bear the antigen identified by FA6-152, whereas the mature BFU-E[33] expressed a low density of this antigen. Mature BFU-E and a large part of the CFU-E were found in the 5% marrow cells whose fluorescent intensity was just located at the threshold of positivity. In contrast, the CFU-E that gave rise to small-sized colonies were all included in the intensively stained fraction. Therefore, the

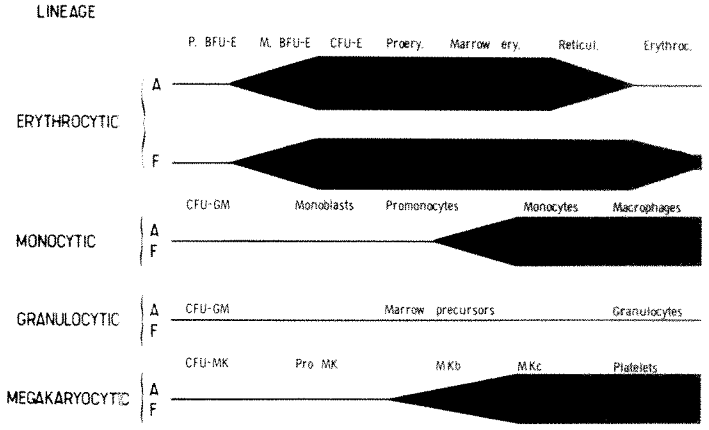

Fig 4. A schematic representation of the expression of FA6-152 on adult and fetal hematopoietic cells. Abbreviations: A, adult; F, fetus.

presence of the antigen defined by FA6-152 on the erythroid progenitors was the reflection of their differentiation stage defining three or four classes of progenitors as previously described when using other differentiation markers such as their hormonal regulation, the size of the colonies, and the in vitro time course of the colonies.[33] Primitive BFU-E whose differentiation depends upon another factor besides Epo were devoid of the antigen defined by FA6-152; mature BFU-E[33] that have acquired an Epo sensitivity bore very low amounts of the antigen, and CFU-E were heterogenous with respect to their antigenic density. The majority of the colonies composed of 32 or more erythroblasts were derived from a progenitor with an antigenic density comparable to the mature BFU-E. In contrast, the colonies composed of eight (or four) to 32 erythroblasts were derived from a progenitor with a high antigenic density. Several authors have pointed out that the compartment of human CFU-E is heterogenous. In particular, the CFU-E that gave rise to small colonies with a peak at day 4 to 5 of culture[34] were highly Epo sensitive[35,36]; they appeared similar to the erythroid progenitor that is intensively stained by the FA6-152 MoAb. EM examination has shown the presence of a homogenous population of undifferentiated cells in the positive fraction. These cells stand as possible candidates for human CFU-E. They have morphologic features similar to the murine CFU-E isolated by a density gradient.[37] In addition, they express another early erythroid marker, ie, carbonic anhydrase I.[27]

Most previously described MoAbs recognized either hematopoietic progenitors, irrespective of their cell lineages,[9,38-43] or CFU-GM.[20,38,44-46] Only one recently described MoAb, ie, EP-1[47] appears specific to the erythroid lineage from the erythroid progenitors (BFU-E and CFU-E) to the erythroblasts. The 5F1 MoAb has also been described as recognizing CFU-E.[38] Therefore, some antibody competition was performed between 5F1 and FA6-152 MoAbs. Only a partial inhibition between the two MoAbs was observed, suggesting that they recognize different epitopes of the same antigen.

In conclusion, we have described a MoAb that recognizes an ontogenic antigen. This antigen is not detected on the surface of adult erythrocytes, but is present on their fetal counterparts. It is present on nucleated erythroblasts and the megakaryocytic and monocytic series. However, for these two hematopoietic cell lineages, FA6-152 is a late marker of differentiation; in contrast, the antigen recognized by FA6-152 is expressed on mature BFU-E with an antigenic density increasing until day 5 CFU-E. This antibody will permit an improved understanding of both normal and leukemic erythroid differentiation.

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Biosynthesis and Secretion of Factor VII, Protein C, Protein S, and the Protein C Inhibitor From a Human Hepatoma Cell Line

By Daryl S. Fair and Richard A. Marlar

Using specific radioimmunoassays, 8 day cultures of Hep G2 cells were shown to contain in their supernatants 16, 74, and 828 ng/mL and in their cell lysates, 8, 55, and 48 ng/2 × 10^8 cells of factor VII, protein C, and protein S, respectively. These proteins and the protein C inhibitor were functionally active, and each of these activities was neutralized by their respective polyclonal antibodies. Although vitamin K had a modest effect, warfarin decreased the activity of secreted factor VII, protein C, and protein S by 50% to 90%. Protein C and protein S antigens were reduced three- to fourfold by warfarin. The protein C inhibitor antigen and activity were unaffected by vitamin K or warfarin treatment. Intrinsic labeling and immunoprecipitation indicated that factor VII, protein S, and the protein C inhibitor were secreted as 52,000, 77,000, and 58,000 molecular weight (mol wt) proteins, respectively. Protein C was secreted as a single-chain protein of about 65,000 mol wt, indicating that all of the vitamin K-dependent proteins are translated and secreted as single-chain molecules. Each of the four proteins studied represented their plasma protein counterparts structurally, functionally, and immunochemically. Thus, all of the known soluble components of the protein C pathway are produced by liver parenchymal cells.

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VITAMIN K-DEPENDENT proteins are characterized as glycoproteins that are carboxylated at selected glutamic acid residues (gla) by a posttranslational process involving at least two enzymes and vitamin K.[1,2] Warfarin is an antagonist of vitamin K and inhibits this reaction. The gla residues permit the binding of calcium, which induces a conformational change in the protein and mediates its binding to negatively charged phospholipid surfaces. Most, but not all of these proteins, contain a second unique alteration, namely, the presence of a β-hydroxyaspartic acid.[3,4] The function of this modified residue is unknown.

Factor VII, a single-chain 50,000-molecular weight (mol wt) protein containing nine gla residues,[5,6] initiates coagulation when associated with the lipophilic, nonenzymatic cofactor, tissue factor.[7] It is an active zymogen exhibiting 0.8% to 4% of the activity of its activated serine protease form.[6,8,9] In contrast, protein C, a two-chain 62,000-mol wt molecule containing 11 gla and one β-hydroxyaspartic acid residues is an inactive zymogen.[3,4,10,11] However, once activated by thrombin-thrombomodulin, it is a potent anticoagulant serine protease that inactivates factors Va and VIIIa.[12] The function of activated protein C is enhanced in the presence of another vitamin K-dependent molecule, protein S.[13] This single-chain 75,000-mol wt protein, which contains nine to ten gla residues and one β-hydroxyaspartic acid residue has been proposed to function as a nonenzymatic cofactor for activated protein C.[3,4,13-15] Inhibition of activated protein C is by a novel, recently isolated 57,000-mol wt single-chain plasma protein that neutralizes activated protein C by forming a stable 1:1 molar complex.[16,17]

The liver has been shown to be the primary source of plasma proteins including those of the coagulation and fibrinolytic systems, although this conclusion was based on indirect evidence of earlier studies.[18,19] With the advent of animal organ perfusion and cell culture, activity studies were used as evidence for the liver and the parenchymal cell as the principal site of synthesis of most of the plasma proteins.[20-25] Because the human molecules have been isolated and well characterized, the human hepatoma cell line, Hep G2, has been used to assign the site of synthesis of most of the zymogens and inhibitors of the coagulation and fibrinolytic systems to the hepatocyte.[26-30] Factor VII has been shown to be produced by rodent liver cells.[21,22,31-33] It has been assumed that most of the components of the protein C system (protein C and protein S) are produced by the liver based on clinical studies.[34,35] We used the Hep G2 cell line to demonstrate that factor VII, protein C, protein S, and the protein C inhibitor are synthesized by hepatocytes and that each of the molecules represents their plasma protein counterparts structurally, immunochemically, and functionally. In addition, we demonstrate that protein C is secreted as a single-chain molecule, analogous to factor X.[30]

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line, Hep G2, was obtained from Drs Barbara B. Knowles and David A. Aden of the Wistar Institute, Philadelphia. The cells were maintained in T-75 culture flasks containing Eagle's modified medium supplemented with 10% heat-inactivated fetal calf serum (FCS) by passage of at least 10^6 cells per flask as previously described.[30] Cells were occasionally cultured for four to five days in the absence of serum to test the effects of 1 μg/mL of a warfarin derivative, 3(α-acetonyl benyl)-4-hydroxycoumarin (Sigma Chemical Co, St Louis) or 10 μg/mL of vitamin K (Aqua Mephyton, Merck, Sharp and Dohme, West Point, Pa) as described previously.[30]

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Proteins. Factor VII,[36] protein C,[10,37] protein S,[15] and the protein C inhibitor[16] were isolated as previously reported. All proteins were homogenous and showed the characteristics of published preparations as judged by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis with and without a reducing agent.[38]

Antisera and neutralization. All antisera used in this study were generated in rabbits as previously reported.[36] For neutralization studies, the IgG fraction of serum was isolated as previously described.[36] Supernatants from four- and five-day serum-free cultures were concentrated 20-fold by ultrafiltration. A constant volume of supernatant was mixed with equal volumes of varying concentrations of specific antibody, and after incubation for 15 minutes at 37 °C, the samples were assayed for biologic activity. Activity values were expressed as percentages of pooled normal human plasma (NHP). Immunopurification of antibodies was accomplished by affinity chromatography on columns constructed of protein C, protein S, or factor VII coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ).

Immunoassays. Double antibody competitive inhibition equilibrium radioimmunoassays were constructed for factor VII,[36] protein S,[35] and protein C. Proteins were radioiodinated by the iodogen method to specific activities of 1.5 to 7.4 μCi/μg.[39] Briefly, to 250 μL of labeled protein (0.5 nmol/L) was added 250 μL of competitor (protein, plasma, or culture supernatant) and 250 μL of antibody diluted to give between 25% to 50% binding of the iodinated ligand. Following incubation at 4 °C for 16 to 20 hours, 250 μL of goat antirabbit IgG was added and incubated at 4 °C for an additional six hours. The immunoprecipitates were collected by centrifugation at 2,000 g for 15 minutes, and 500 μL of the supernatant was counted for radioactivity in an Iso-Data 20/20 series gamma counter (Iso-Data Inc, Palatine, Ill). The buffer used for the assay contained 42 mmol/L borate, pH 8.3, 25 mmol/L NaCl, 10 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 10 U/mL Trasylol, 1.25% heat-inactivated normal rabbit serum, and 0.02% sodium azide.

Protein C and protein C inhibitor concentrations were determined using a radiolabeled Laurell electroimmunoassay, and protein S was assayed by a Coomassie-stained Laurell rocket assay.[40,41] The IgG fraction of antiserum was isolated and radiolabeled with Na ^{125}I (Amersham Corp, Arlington Heights, Ill) by the chloramine T method.[40] Antiserum to protein C or its inhibitor was supplemented with labeled antibody (1 × 10^6 cpm) and added to 0.9% agarose in Tris-barbital buffer, pH 8.4. Samples (8 μL) were electrophoresed overnight at a constant current (10 mA/per slide) at ambient temperature. Slides were washed extensively, dried, and autoradiographed. The same procedure was used for protein S, except labeled IgG was not included and the slides were stained with Coomassie blue after extensive washing. The log concentration of the protein being assayed was linearly correlated to the height of the rocket. The unknown samples were expressed as a percentage of a NHP pool.

Activity assays. Factor VII activity was determined in a one-stage coagulation assay.[36] To 50 μL of plasma immunochemically depleted of factor VII was added 50 μL of plasma diluted in 20 mmol/L tris(hydroxymethyl) aminomethane (Tris) (pH 7.4) containing 140 mmol/L NaCl (TBS) and 1 mg/mL bovine serum albumin (BSA) or 20-fold concentrated Hep G2 supernatant. After incubation for one minute at 37 °C, 100 μL of prewarmed rabbit brain thromboplastin containing 12.5 mmol/L $CaCl_2$ (Simplastin, General Diagnostics, Morris Plains, NJ) was added and the time for clot formation determined. Activities were quantitated and presented as a percentage of pooled NHP.

Human protein C was assayed for its chromogenic activity by the method of Comp et al.[42] Thrombin-thrombomodulin was used to activate protein C, and anti–protein C IgG-Sepharose was used to isolate the activated molecules. The activity of activated protein C was determined using the synthetic substrate S2238 (Helena Laboratories, Beaumont, Tex) as described previously.[40,42] The concentration of protein C in the sample correlated with the linear change in absorbence. The protein C inhibitor activity was assayed as described by Marlar et al.[40] An inverse relationship between the absorbence generated by a fixed amount of activated protein C and the concentration of protein C inhibitor was found and used for construction of a standard curve. Protein S activity in the conditioned medium was assayed by the method of Comp et al.[43] There was a linear relationship between the clotting time and the concentration of protein S. The levels of each factor were expressed as a percentage of a NHP pool.

Immunoprecipitation. Cultures of Hep G2 cells were grown for ten days, washed, and incubated with 4 mL of methionine-free minimum essential medium containing 1 mmol/L benzamidine, 10 μg/mL vitamin K, 1 U/mL heparin (Sigma Chemical Co.) and 150 μCi/mL of ^{35}S-methionine (Amersham Corp). After five to six hours, the supernatants were collected and clarified by centrifugation at 100,000 g for 45 minutes at 4 °C. The supernatants were supplemented with BSA (5 mg/mL) and cleared using *Staphylococcus aureus* previously washed in immunoprecipitation buffer (IPB; 12.5 mmol/L borate, pH 8.3, containing 7.5 mmol/L NaCl, 0.05% Tween 20, 0.5% Triton X-100, 1% Nonidet P-40, 10 mmol/L benzamidine, 1 mmol/L PMSF, 5 U/mL Trasylol, and 0.02% NaN_3).[30] Aliquots were incubated with immunopurified antibodies or the IgG fraction (5 to 10 μg) of normal serum or antisera to factor VII, protein C, protein S, or protein C inhibitor in the presence of 25% (vol/vol) IPB. To confirm a precipitated band as a specific protein, we included 50 μg of the purified protein to compete with the labeled molecule for the specific antibody. After incubation overnight at 4 °C, the immune complexes were collected by the addition of *S. aureus*, incubated for 30 minutes at room temperature and centrifuged in a microfuge for three minutes. The pellets were washed as described previously,[30] and the SDS-soluble material was electrophoresed on SDS–9%-polyacrylamide slab gels[44] containing ^{14}C-protein standards (New England Nuclear, Boston) in the absence and presence of 1% 2-mercaptoethanol. Slab gels were fixed in 10% acetic acid, 25% methanol for one hour, washed extensively with water, incubated with 1 mol/L sodium salicylate for 30 minutes, dried, and subjected to autoradiography.

RESULTS

Cell extracts and supernatants from eight-day cultures were analyzed for the presence of factor VII, protein C, and protein S using sensitive and specific double-antibody–competitive equilibrium radioimmunoassays. The supernatants contained an average of 16.3, 74.0, and 828 ng/mL of factor VII, protein C, and protein S, respectively. Cell extracts were prepared by incubating 7 × 10^8 cells in 1 mL of 1% Triton X-100 containing 10 mmol/L EDTA for 30 minutes at 37 °C and diluting to 2 × 10^8/mL with radioimmunoassay buffer. Factor VII, protein C, and protein S were measured at 8.5, 54.5, and 48.1 ng/mL, respectively. Hence, all three proteins were detected as secreted products and intracellular proteins, provided a sufficient number of cells were used.

To determine if our radioimmunoassay could accurately quantitate the proteins present in the supernatants and cell extracts of Hep G2 cells, we compared the ability of purified proteins, NHP, and a ten-day Hep G2 supernatant to inhibit the binding of iodinated molecules to their respective antibodies. Figure 1 depicts a radioimmunoassay specific for protein C and shows that the protein C in plasma secreted by the cells was immunochemically identical to the purified molecule, as evidenced by the parallel lines of inhibition. Both the plasma and the supernatant did not completely inhibit the reaction, because of the relatively low concentrations of protein C in these preparations. FCS did not compete, indicating that bovine and human protein C are immunochemically distinct. Analogous results were obtained with the radioimmunoassays for factor VII and protein S. Only protein S showed some immunologic cross-reactivity between the bovine and human molecules as reported by others[14,15] and was corrected for in the quantitation. Thus, our assays could accurately measure these three proteins secreted from the cells.

To determine the secretion rates of the vitamin K–dependent proteins from the Hep G2 cells, three T-75 cultures were each seeded with one million cells to give maximal rates of secretion[30] and incubated with 10 mL of culture medium containing serum. On even numbered days, 2 mL of culture medium was removed from each flask and replaced with an equivalent volume of fresh medium. The samples were then quantitated for factor VII, protein C, and protein S by radioimmunoassay, and these values were corrected for the dilution by added medium. Figure 2 shows the increase in each of the proteins with time of culture. However, the absolute amounts of each protein differed with factor VII the least and protein S the greatest. The maximum rates of protein accumulation determined between day 6 and 10 was 5, 25, and 170 ng/mL/d for factor VII, protein C, and protein S, respectively.

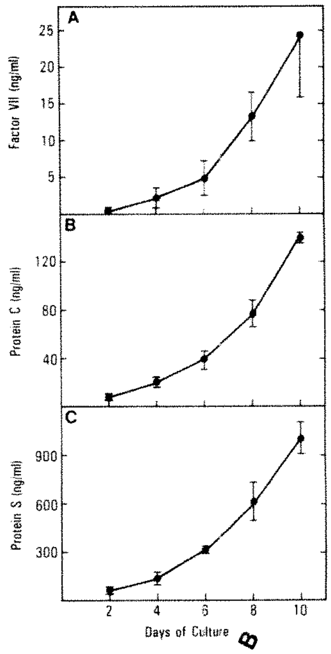

Fig 2. Time course of the accumulation of factor VII (top), protein C (center), and protein S (bottom) in Hep G2 supernatants. Three T-75 flasks were each seeded with 1 × 10^6 Hep G2 cells in the presence of 10 mL of MEM containing 10% FCS. On the days indicated, 2 mL of medium were removed and replaced with fresh medium. Antigen was measured using specific radioimmunoassays. The values were corrected for dilution and are plotted as the mean ± SD (error bars).

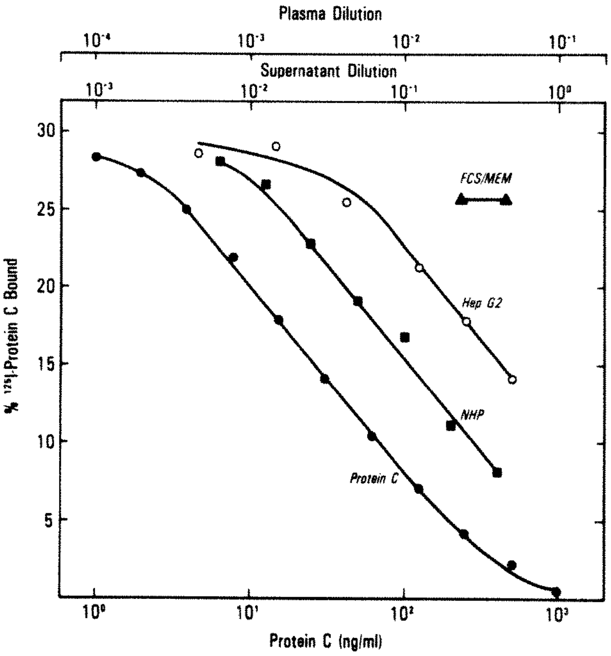

Fig 1. Protein C equilibrium competitive inhibition radioimmunoassay. The binding of ^{125}I-protein C (0.5 nmol/L) to rabbit antiserum (diluted to give 30% binding of the ligand) was inhibited by dilutions of purified protein C (●), NHP (■), ten-day Hep G2 culture supernatant (○) or MEM containing 10% heat-inactivated FCS (▲).

The biologic activity of the secreted proteins was analyzed using specific coagulation assays and compared to the quantity of secreted protein. Activity and antigen values were normalized to NHP for comparison (Table 1). Hep G2 cells were cultured in medium containing serum for 7 days, washed, and cultured an additional four to five days in serum-free medium supplemented with 10 μg/mL vitamin K. The supernatants were collected, clarified by centrifugation, and concentrated 20-fold by ultrafiltration. The activities measured for factor VII, protein C, protein S, and the protein C inhibitor paralleled their respective antigen levels, indicating that the secreted molecules were fully functional with specific activities (activity/antigen) of about 0.96 to 1.23. Further, the activities of each of the four proteins could be specifically neutralized, if the concentrated supernatants were pretreated with their respective antibodies before assay (Table 1). Normal IgG did not significantly affect the function of the four molecules. Thus, these four secreted proteins were biologically active, and their activity could be inhibited by specific antibodies.

Previously, we showed that vitamin K and warfarin modulated the amount of prothrombin secreted by Hep G2 cells.[30] The effect of these agents on the quantity and activity of secreted protein C, protein S, and the protein C inhibitor were studied (Table 2). Seven-day-old cultures were washed and incubated under serum-free conditions in the absence

Table 1. Neutralization of Functional Activities of Factor VII, Protein C, Protein S, and the Protein C Inhibitor Secreted From Hep G2

Protein	Percentage of Antigen*	Treatment†	Percentage of Activity*
Factor VII	4.7	buffer	5.8
		normal IgG	5.3
		anti–factor VII	<0.4
Protein C	50	buffer	52
		normal IgG	53
		anti–protein C	<3
Protein S	75	buffer	75
		normal IgG	72
		anti–protein S	<3
Protein C inhibitor	100	buffer	102
		normal IgG	106
		anti–PCI	<3

*Activity and antigen values are presented as percentages of pooled NHP.

†Supernatants were incubated with buffer, normal, or immune IgG (40 nmol/L) for 15 minutes at 37 °C and then assayed for activity.

Serum-free culture medium from Hep G2 cells was concentrated 20-fold by ultrafiltration.

and presence of 10 μg/mL vitamin K or 1 μg/mL warfarin for an additional four days. The concentration of these proteins remained constant (protein C) or increased slightly (protein S) in the presence of vitamin K. When the cells were cultured in the presence of warfarin, a three- to fivefold reduction in the total accumulated protein C and protein S was observed by electroimmunoassay (Table 2) or radioimmunoassay (data not shown). Accumulation of the protein C inhibitor (Table 2) and the total secreted protein (data not shown) were unaffected by either treatment. Functional activity of protein C, protein S, and factor VII was decreased by 50% to 90% in the presence of warfarin, whereas the protein C inhibitor activity was not affected. Also, protein C and protein S activities were disproportionately lower than their respective antigen values. For protein C, a tenfold reduction of activity was observed compared to only a threefold reduction in antigen. The protein S activity decreased fourfold, whereas the antigen only decreased 2.5-fold in the presence of the vitamin K antagonist.

Table 2. The Effect of Warfarin and Vitamin K on the Secretion and Activity of Protein C, Protein S, the Protein C Inhibitor, and Factor VII

	Protein C		Protein S		PC Inhibitor		Factor VII
	Act*	Ag*	Act	Ag	Act	Ag	Act
Control	45	42	60	67	85	86	25
Vitamin K	45	47	77	75	86	80	23
Warfarin	3	16	15	28	88	80	10

*Antigen (Ag) quantitation was by electroimmunoassay and activity (Act) was measured in coagulation or amidolytic assays as described in Materials and Methods. Values are presented as the percentage of normal human plasma for each individual protein.

T-75 flasks were cultured for seven days in medium containing 10% FCS. After washing, the cells were incubated for four days in the presence of MEM (control), medium containing 10 μg/mL vitamin K or medium containing 1 μg/ml warfarin. Supernatants were concentrated 20-fold by ultrafiltration before analysis.

To demonstrate the synthesis of these proteins by the Hep G2 cells, cultures of 10-day-old cells were washed and incubated with methionine-free MEM containing 150 μCi/mL of ^{35}S-methionine for six hours at 37 °C. After centrifugation, the supernatants were immunoprecipitated and electrophoresed on SDS-polyacrylamide slab gels in the absence and presence of a reducing reagent. In selected experiments, excess unlabeled protein was added to the supernatant as a competitor before immunoprecipitation. Figure 3 depicts autoradiographs of the slab gels under reduced conditions. Factor VII and protein S were precipitated as single-chain species with apparent mol wts of 52,100 ± 1,600 and 77,300 ± 1,000, respectively. The antibody to the protein C inhibitor was known to recognize at least one other plasma protein. Therefore, to identify which of the components represented the inhibitor, we included 50 μg of highly purified protein C inhibitor in the immunoprecipitation reaction and looked for the mol wt band diminished in the presence of the added protein. Of the two major bands observed in the gel, only one was displaced by added inhibitor and corresponded to an apparent mol wt of 58,000 ± 800. Two minor bands of about 98,000 and 77,000 also appeared to decrease with addition of excess protein C inhibitor. Protein C migrated as a single, diffuse component with a mol wt of approximately 65,000 ± 3,000, indicating that protein C was secreted as a single-chain molecule. The addition of

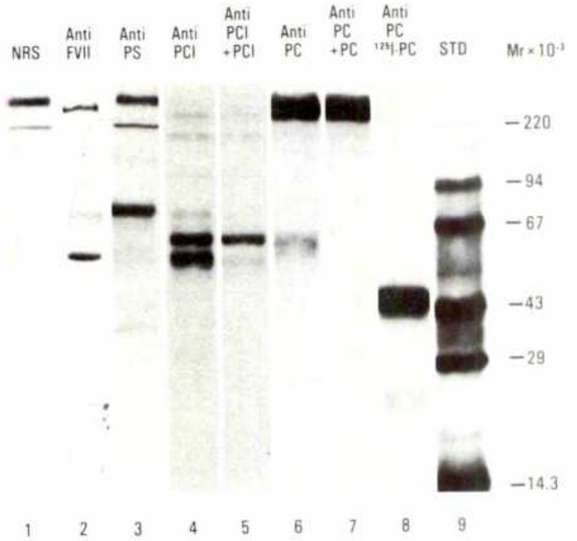

Fig 3. Autoradiogram of the immunoprecipitates from ^{35}S-methionine-labeled proteins secreted from Hep G2 (lanes 1 to 7) and ^{125}I–protein C (lane 8) following incubation with normal rabbit IgG (lane 1), anti-factor VII (lane 2), anti–protein S (lane 3), anti–protein C inhibitor (lanes 4 and 5) or anti–protein C (lanes 6 to 8), and precipitation with *S. aureus* and SDS polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. Purified protein C inhibitor (50 μg; lane 5) or protein C (50 μg, lane 7) was added to the supernatants before immunoprecipitation. Standards used for quantitation (lane 9) contain myosin (220,000), phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), and lysozyme (14,300).

excess purified protein C before immunoprecipitation prevented recovery of this band. We did not observe mol wt bands in these precipitates that would correspond to the heavy and/or light chain of protein C. For comparison, iodinated purified protein C was added to the medium and immunoprecipitated under the same conditions as the Hep G2 supernatants. The characteristic two-chain form of protein C was seen with apparent mol wts of 45,000 ± 700 and 25,500 ± 2,000 for the heavy and light chains, respectively. Although the heavy chain is clearly evident, the light chain was much fainter, indicating a disproportionate iodination of the polypeptides. However, prolonged exposure time facilitated the detection of the light chain.

DISCUSSION

The Hep G2 cell has been shown to synthesize and secrete many of the proteins thought to be derived from the liver.[26-30,45,46] The molecules of the coagulation and fibrinolytic systems demonstrated to be the product of this cell reflect their plasma counterparts structurally, immunochemically, and functionally.[26-30] In this report we have extended these studies to include molecules that can initiate (factor VII) and regulate (protein C, protein S, and protein C inhibitor) the coagulation process. In each case, these molecules were biologically active, neutralized by specific antibodies, modulated by known pharmacologic agents, and were structurally and immunochemically similar to the molecules isolated from plasma. Thus, this study provides the first direct evidence that the soluble components of the protein C pathway are produced by the liver and confirms the synthesis of factor VII by hepatocytes.

The finding that protein C is synthesized and secreted as a single-chain molecule is consistent with recently reported data from recombinant DNA (cDNA) cloning indicating that protein C is transcribed as a single contiguous messenger RNA (mRNA),[47,48] and from immunoblotting experiments suggesting that 10% of plasma protein C is present in a single-chain form.[49] Recently, single-chain protein C has been isolated from plasma and has the same activation and enzymatic properties as the two-chain form.[50] Similar findings have been reported for factor X, the only other two-chain vitamin K–dependent protein.[25,30,51-53] Thus, all of the molecules in this protein family appear to be the products of individual genes. Because we immunoprecipitate only secreted material, protein C and factor X must undergo a postsecretion processing event leading to the generation of their respective two-chain plasma forms. The nature of this modification remains to be elucidated. It is possible that a minor population of protein C is processed during secretion. However, prolonged exposure of our gels did not reveal the presence of any other mol wt bands that could be specifically competed by addition of purified protein C before immunoprecipitation. Thus, within the limitations of this analysis, only single-chain protein C is secreted from the Hep G2 cells.

Synthesis of factor VII by the liver has been demonstrated using rodent organ perfusates, preparation of liver microsomes, and the use of established rat cell lines.[21,22,31-33] All of these investigations have been limited to the use of activity measurements in coagulation assays to make their conclusions. Because human factor VII has been isolated in sufficient quantities and monospecific antibodies made to it,[36] the appropriate reagents were in hand to unequivocally establish the human hepatocyte as a site of origin of this molecule. Characteristics of the secreted protein were very similar to those of the isolated molecule with a mol wt of about 50,000[5,6,36] and a similar immunochemical profile. Extrapolation to normal tissue from the findings in a tumor cell must always be guarded; however, very similar correlations between proteins isolated from plasma and characterized from this cell line make our conclusions tenable.

Protein S and the protein C inhibitor shared the same properties as their isolated plasma counterparts. Protein S had an apparent mol wt of 75,000 to 77,000 under reduced conditions, indicating that it was synthesized and secreted as the mol wt form originally reported[14] but was smaller than the more recently published measurements of 84,000 daltons by Dahlback.[15] We saw no evidence of proteolysis since mol wt estimates in reduced and nonreduced gels were similar and no degradation was seen if iodinated protein S was added to cell lysates. Immunochemically and functionally, the secreted molecule was identical to the isolated plasma form. Secretion of protein S from these cells appears very efficient when compared to the other vitamin K–dependent proteins. The quantity of intracellular protein S is less than that measured for prothrombin, and the rate of secretion is about 50% greater. This may be attributed to different secretion mechanisms or to differences in culture conditions (ie, serum lots) and age of the cells (about 30 more passages in this study relative to the prothrombin analysis). Both protein C and factor VII have high secretion rates relative to factor X. This may also reflect a normal process since both protein C and factor VII have shorter half-lives and, therefore, would require higher synthetic rates to maintain a steady state. The protein C inhibitor was also very similar to its recently reported plasma counterpart functionally, immunochemically, and structurally.[16,17] Thus, all the soluble components of the protein C pathway are produced by the hepatocyte.

One of the principal characteristics of the family of vitamin K–dependent proteins is the effect of warfarin-like drugs on the synthesis of these molecules. Previously, we demonstrated that prothrombin and factor X, but not antithrombin III, were accumulated in the culture medium at a lower rate than untreated cells.[30] Protein C and protein S were shown to be similarly affected, with a reduction in the amount of each protein by about three- to fourfold. Protein C inhibitor, a protein thought not to be modulated by these drugs, was unaffected in the amount of secreted antigen and activity. The functions of protein C, protein S, and factor VII were significantly decreased in the presence of warfarin, and this reduction was greater than that observed for accumulated antigen. This indicates a decrease in the specific activity of the secreted molecules and is consistent with the findings obtained from patients undergoing warfarin therapy.[34-36] Vitamin K did not significantly increase production of these molecules as observed with prothrombin.[30] It is possible that prothrombin synthesis is more sensitive to vitamin K or that the concentration of vitamin K in our lot of FCS was optimal to produce and maintain a maximal rate of synthesis over the serum-free culture period. This latter suggestion is supported by the production of fully active proteins by cells not supplemented with exogenous vitamin K. Thus, our results suggest that the decrease in the vitamin K–dependent proteins (measured by antigen) present in the plasma of individuals undergoing warfarin therapy can be attributed to at least a decrease in the synthetic rate of these molecules. Increases in the catabolic rates may also occur and need to be investigated.

The human hepatoma cell line, Hep G2, has been of benefit in our determining the origin of a number of plasma proteins. This cell line has been shown to synthesize and secrete most of the vitamin K–dependent proteins. Factor IX is not transcribed (P. Tolstoshev, personal communication, Transgene, Strasbourg, France, January 1985) or translated by this cell,[30] and protein Z is present in culture supernatants at very low concentrations (D. Fair, unpublished observations, June, 1984). In addition, this cell has the capacity to synthesize and secrete most of the proteins of the coagulation, fibrinolytic, and protein C systems including their inhibitors. Because the cell line responds normally to known pharmacologic modulators, it provides an excellent model to investigate the molecular events underlying these measured responses.

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NOTE ADDED IN PROOF

After submission of our manuscript Morito et al (Biochim Biophys Acta 844:209, 1985) published the synthesis of the protein C inhibitor by Hep G2 cells. However, these investigators were unable to detect protein C in cell supernatants in contrast to our findings.

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Purification of Human Erythropoietin to Homogeneity by a Rapid Five-Step Procedure

By Gerald Krystal, H. Robert C. Pankratz, Neal M. Farber, and John E. Smart

Human urinary erythropoietin (Ep) has been purified using a simple five-step procedure to yield preparations with potencies of 80,000 U/mg in 25% yield. The five steps involve: (1) affinity chromatography on CM Affi-Gel Blue, (2) chromatofocusing, (3) wheat germ lectin (or hydroxylapatite) chromatography, (4) reverse-phase high-performance liquid chromatography (HPLC) using a phenyl column, and (5) preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ep activity was determined at each stage using a highly sensitive and specific in vitro assay that measures [^{3}H]-thymidine incorporation into erythroid cells from spleens of phenylhydrazine-treated mice. The step 5 material was also tested with the in vivo polycythemic mouse assay procedure and was found to have a similar potency to that obtained in the [^{3}H]-thymidine in vitro assay. SDS-PAGE analysis of the step 5 material revealed a single 38.5-kd band that co-migrated with Ep bioactivity. Homogeneity was confirmed by amino acid sequence analysis. Starting with urine containing ~13 U/mg of protein, the cumulative degrees of purification achieved with each step were: step 1, 25-fold; step 2, 75-fold; step 3, 300-fold; step 4, 1,500-fold; and step 5, 5,000-fold. Corresponding overall recoveries after each step were: >100%, 70%, 45%, 30%, and 25%. These recoveries could be obtained when as little as 5,000 U of starting urinary Ep were processed because of the introduction of Tween 20 and SDS into buffers used at various stages of the purification procedure. In addition, a rapid method for determining Ep purity which involves reverse-phase HPLC of trypsinized ^{125}I-labeled Ep is presented. This allows the establishment of purity with far less material than is required for amino acid sequencing.

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IT IS NOW well established that erythropoietin (Ep) plays a major role in regulating the terminal phases of erythropoiesis.[1,2] However, very little is known about the mechanism of action of this hormone, owing, in large part, to difficulties historically encountered in acquiring sufficient quantities of highly purified preparations. In 1977, Miyake et al were the first to publish a procedure for purifying human Ep to homogeneity using classical biochemical techniques.[3] This method used eight lengthy steps and gave only reasonable yields (15%) when very large amounts of starting material were used, ie, 2,500 L of starting urine containing 7 million U of Ep. This amount of starting material is not available to most laboratories involved in Ep research. Recently, Yanagawa et al reported a simple two-step procedure for purifying Ep to homogeneity that involved an immunoadsorbent column of monoclonal antibodies to sodium dodecyl sulfate (SDS)-denatured Ep.[4] Although this procedure represents a major advance over the original approach, it is contingent upon access to a suitable monoclonal antibody. Such antibodies are not yet widely available, and alternative methods thus continue to be of interest.

In this report, we describe a simple five-step protocol for purifying Ep to homogeneity that yields a 25% final recovery even when as little as 5,000 U of starting urinary Ep are used. We believe this is possible because of the introduction of ionic (ie, SDS) and nonionic (ie, Tween 20) detergents into the buffers used at various stages of the purification procedure. This appears to reduce the adsorption of Ep to glass and plastic surfaces markedly.

We also describe a rapid procedure for determining Ep purity that involves reverse-phase high-performance liquid chromatography (HPLC) of trypsinized ^{125}I-labeled Ep. Using this procedure, pure Ep yields two clearly defined peaks of radioactivity. This therefore allows the establishment of Ep homogeneity without the need for amino acid sequence analysis.

MATERIALS AND METHODS

Materials

Polybuffer exchanger 94 (PBE94), polybuffer 74 (PB74) and wheat germ Lectin-Sepharose 6MB (~5 mg lectin/mL sedimented gel) were purchased from Pharmacia Fine Chemicals, Montreal. CM Affi-Gel Blue, hydroxylapatite (Bio-Gel HTP), and the electrophoresis purity reagents, acrylamide, bis acrylamide, TEMED, and ammonium persulfate were obtained from Bio-Rad, Richmond, Calif. The μBondapak phenyl HPLC column (0.39 × 30 cm) was acquired from Waters Associates, Milford, Mass. The Spectra-Physics 8000 HPL chromatograph was purchased from Technical Marketing Associates, Vancouver, Canada, and the UA-5 absorbance/fluorescence monitor used for HPLC was obtained from Isco, Lincoln, Neb. Insoluble neuraminidase (*N*-2382), polyethylene glycol (PEG) 4000 and 6000, Triton X-100 (TX100), sodium deoxycholate (DOC) (D-6750), Tween 20 (monolaurate polyoxyethylenesorbitan), human transferrin (T-2252), bovine serum albumin (BSA) (A-7638), and silver nitrate were obtained from Sigma, St Louis. Nonidet P40 (NP40) was purchased from Particle Data Laboratories Ltd, Elmhurst, Ill; SDS, specially pure, was purchased from BDH Chemicals, Toronto. For HPLC, *n*-propanol (spectrophotometric grade) was purchased from Aldrich Chemical Co, Milwaukee.

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Urine Collection and CM Affi-Gel Blue Chromatography

Urine was collected from aplastic anemic patients whose urinary Ep titers had been previously established as >0.5 U/mL. The urine was frozen immediately after excretion and was stored at −15 °C. Subsequent preparation for CM Affi-Gel Blue chromatography and the details of this first step in the purification procedure have been described previously.[5]

Assay for Ep

A microassay procedure for Ep, based on the incorporation of $[^3H]$-thymidine into spleen cells from phenylhydrazine-treated mice,[6] was used throughout this study. Ep content of fractions obtained at different stages of purity were determined by comparison with either the World Health Organization (WHO) standard preparation No. 2[7] or CM Affi-Gel Blue purified human urinary Ep (100 to 350 U/mg)[5] calibrated against the WHO standard.

The Ep activity present in the purest preparation (step 5 Ep) was also determined in vivo using the polycythemic mouse assay (five mice per sample).[8]

Protein Determination

Protein concentrations for all but the last step in the purification procedure were measured using the Coomassie Brilliant Blue G-250 dye-binding technique of Bradford with BSA as the protein standard.[9] For the final electrophoretically pure Ep preparation, protein content was determined by silver-staining analytical SDS polyacrylamide gels using a modified Ohsawa and Ebata procedure,[10] scanning the gels using a Hoefer Scientific GS300 Transmittance/Reflectance scanning densitometer (San Francisco), and comparing the Ep area to known protein standards.

Stabilization Studies

Under sterile conditions, step 2 Ep (refer to Table 1 for definition of steps) at 2,750 U/mg; 1,100 U/mL was diluted to 500 mU/mL with PBS [10 mmol/L of phosphate-buffered saline (PBS)]. Immediately following this dilution, 100-μL aliquots were dispensed into 12 × 75 mm tubes (Falcon, Oxnard, Calif) containing either 10 μL of 10% bovine serum albumin (BSA); 1% SDS, 0.2% Tween 20, 1% BSA, 10% polyethylene glycol (PEG) 6000, 0.1% NP40, 0.1% TX100, 0.1% DOC, or PBS. Concentrated step 2 Ep (1,100 U/mL) was also diluted to 500 mU/mL with 0.15 mol/L of NaCl, and 100 μL was added to a Falcon tube containing 10 μL of 100 mmol/L of $CaCl_2$. Control tubes were also prepared as above but without Ep. Immediately after the dispensing, the two tubes (with and without Ep) containing 10 μL of 10% BSA were frozen, and the remaining tubes were incubated for 12 hours at 23 °C. For the in vitro assay, aliquots were either tested at a final concentration of 20% (ie, 20 μL/100 μL total vol), or were diluted tenfold, with 0.1% BSA in α minimal essential medium (MEM) without nucleosides and were tested at a final concentration of 2%.

Analytical SDS-PAGE

To assess the electrophoretic purity at each stage of the purification procedure, samples were made at 2% with SDS, 5% with 2-mercaptoethanol, and 10% with glycerol, and were heated to 100 °C for two minutes. They were then subjected to standard SDS-PAGE as described by Laemmli[11] using either 8%, 10%, or 12% acrylamide running gels, 0.85-mm thick by 14-cm long. Following electrophoresis, some gel lanes were sliced into 0.5-cm sections, placed in 1.5-mL polypropylene microcentrifuge tubes (VWR Scientific Inc, San Francisco), and rocked overnight at 4 °C with 0.5 mL of 0.02% Tween 20 in PBS. Microfuged supernatants were then assayed for activity using the $[^3H]$-thymidine assay procedure.[6] The rest of the gel lanes were stained using a modified Ohsawa and Ebata silver-staining procedure.[10]

Tryptic Fragment Preparation and Analysis

Approximately 100 ng of step 5 Ep in 50 mmol/L of sodium phosphate (Ph 7.4), 1% SDS was iodinated with 1 mCi $[^{125}I]$-NaI (NEN) using 0.5 mg/mL of chloramine T[12] for three minutes at room temperature. The 25-μL reaction mixture was then treated with 5 μL of 5 mg/mL sodium metabisulfite and 50 μL of 2 mg/mL KI and was passed over a 15 × 0.7 cm Sephadex G-25 (medium) column equilibrated with 0.1% SDS. The peak fractions were pooled, lyophylized, and resuspended in 80 μL of Laemmli loading buffer, boiled for five minutes, and electrophoresed on a 15% SDS-polyacrylamide gel. The region of the gel containing the labeled protein was visualized by one-hour autoradiography of the wet gel. The protein band was excised, and the gel piece was shaken in 50 mL of 40% methanol, 10% acetic acid for two hours with one change, to remove SDS. The gel was then minced, lyophilized, and resuspended in 5 mL of 0.1 mol/L of ammonium bicarbonate. Five microliters of 10 mg/mL TPCK trypsin (Worthington Biochemicals, Millipore Corp, Bedford, Mass) was added, and digestion proceeded for 18 hours at 37 °C. Another 5 μL of trypsin was added for another two hours at 37 °C. The digested fragments were washed through a 0.45-μm millipore HA filter with 0.1 mol/L of ammonium bicarbon-

Table 1. Purification of Human Urinary Ep

Step	Vol (mL)	Protein Concentration (mg/mL)*	Ep Concentration (U/mL)	Specific Activity (U/mg)	Total Units (U)	Overall Yield (%)
Starting urine	23,600	0.07	0.95	13.4	22,420	100
1. CM Affi-Gel Blue concentrate	91	0.82	280	340	25,500	114
2. Chromatofocusing concentrate	9.0	0.89	1,750	1,970	15,700	70
3. Wheat germ concentrate	2.2	1.10	4,600	4,180	10,120	45
4. Phenyl-HPLC concentrate	4.0	0.08	1,650	21,000	6,600	30
5. Preparative SDS-PAGE	14.0	—	400	80,000†	5,630	25

Ep, Erythropoietin; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

*Protein concentrations were determined using the Coomassie Brilliant Blue G-250 dye-binding technique.[8]

†Deduced from gel-scanning analyses of silver-stained gels.

ate and lyophilized. For the reverse-phase chromatography of tryptic peptides, the peptides were dissolved for ~ten minutes in 100 μL of 90% formic acid at 23 °C and were then diluted with 400 μL of H_2O. The sample was then applied to a 4.6-mm × 25-cm Spherisorb 10 ODS (C_{18}) reverse phase column (Spectra-Physics). The column was run at 40 °C and at a flow rate of 1.4 mL/min. After injection of the sample, the column was washed for five minutes with 4.5% formic acid (PSC Reagents, J.T. Baker, Phillipsburg, NJ) and was then developed with a linear gradient from 0% to 62.5% ethanol (Photrex Reagents, J.T. Baker) in 4.5% formic acid over a period of 95 minutes on a Beckman 345T HPLC system. Fractions of 0.7 mL were collected in 0.5 minute each, yielding a total of 200 fractions; each fraction was counted in a Beckman 5500 gamma counter.

Amino Acid Sequencing

Approximately 10 μg of step 5 Ep was dialyzed against 0.01% SDS, lyophilized, and resuspended in 100 μL of H_2O. The sample was boiled, and 10 μL was removed for amino acid analysis. The remaining aliquot was sequenced on an Applied Biosystems Gas Phase Sequencer model 470A using MeOH/HCl conversion. The PTH amino acids were analyzed on a Hewlett Packard 1084B HPLC with model 1040A diode array detector.[13]

RESULTS

"Stabilization" Studies

During our initial studies into the purification of Ep, we discovered that substantial losses in activity were incurred as homogeneity was approached. This was particularly evident when purifications were carried out with <10,000 U of starting material (human urine). Subsequent experiments suggested that losses were owing, in large part, to sticking of Ep to glass and plastic surfaces, and that this adsorption could be prevented by the addition of 0.1% BSA or 0.1% SDS to our Ep preparations.[5] Evidence is now presented, following a more extensive survey of potentially "stabilizing" compounds, that 0.02% Tween 20 is as effective as BSA or SDS for maintaining bioactivity of partially purified Ep preparations (Table 2). One percent PEG 6000, 0.01% NP40, and 0.01% TX100 were also found to be effective, although they were less effective than either Tween 20, BSA, or SDS. Little or no protective effect was observed with 0.01% DOC or 10 mmol/L of calcium chloride. Moreover, TX100 and NP40 were toxic in the phenylhydrazine mouse spleen cell assay at final concentrations of >0.001%, whereas SDS was not, at 0.002%; Tween 20 was nontoxic even at concentrations which effectively prevented adsorption of Ep to plastic ware (0.02%). Because Tween 20 was a nonionic detergent and was therefore unlikely to cause significant changes to the native physical properties of Ep, it was added routinely to buffers used in the purification procedures subsequent to the CM Affi-Gel Blue step.

Table 2. Effect of Various Compounds on Ep "Stability"

Conditions of Storage	Ep Activity Recovered (%)
1% BSA and frozen	100
PBS	3.6
PBS (− Ep)	0
0.1% SDS	51*
0.1% BSA	51
0.02% Tween 20	54
1% PEG 6000	28
0.01% TX100	29*
0.01% NP40	36*
0.01% DOC	4.2
10 mmol/L of $CaCl_2$	0*

Ep, erythropoietin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate.

Incubations were carried out as described in Materials and Methods section; samples were assayed, unless otherwise indicated, at a final concentration of 20% using the standard $[^3H]$-thymidine microassay.[5]

*Assayed at a final concentration of 2% because of toxicity at 20%. Controls, without Ep, demonstrated no toxicity at 2%.

Purification Procedure

Aliquots of frozen urines, collected from patients with potentially high Ep titers, were thawed, dialyzed against α MEM without nucleosides, and tested in the $[^3H]$-thymidine suspension culture assay at a final concentration of 10%. Those urines containing Ep levels >0.5 U/mL were used for purification. This preliminary screening was essential since, in our experience, only about one-third of the aplastic anemic patients with hemoglobins of <6 g/dL showed urinary Ep titers significantly >0.01 U/mL. Selected urines were then decanted through four layers of Curity Hospital towels, made 0.025 mmol/L with phenylmethyl sulfonyl fluoride and 0.025% with phenol, and were pressure-filtered through a Sartorius Separator and a type SM 13400 membrane filter (Canlab, Vancouver, Canada) as described previously.[5]

CM Affi-Gel Blue fractionation. This step has already been described in detail.[5] In brief, filtered urine was applied at 4 °C to a CM Affi-Gel Blue column (40 mL/mL gel) preequilibrated with 0.15 mol/L of NaCl and 10 mmol/L of Na phosphate (pH 7.2). Under the conditions used, Ep bound to the column whereas most of the applied protein did not. Ep was subsequently eluted, using a steep salt gradient from 0.15 mol/L to 1.15 mol/L of NaCl in 10 mmol/L of Na phosphate buffer. A typical elution profile is shown in Fig 1. Ep eluted as a broad peak, suggesting heterogeneity, at ~0.9 mol/L of NaCl. The column was then regenerated at 23 °C with 8 mol/L of urea. This fractionation procedure routinely gave a 25-fold to 50-fold purification of Ep, with an apparent Ep recovery in excess of 100%. Typically, specific activities following this step were in the range of 100 to 350 U/mg.

Chromatofocusing. The Ep-containing fractions from CM Affi-Gel Blue chromatography were pooled, concentrated ~tenfold by ultrafiltration using an Amicon Model 402 apparatus (Amicon Corp) fitted with a PM10 membrane, and dialyzed overnight at 4 °C against buffer A (20 mmol/L of Tris-Cl, 0.1% PEG 4000 [pH 7.0]). This step 1 Ep was then applied to a PBE94 column (6,000 U/mL of gel) preequilibrated with buffer A. Five-milliliter fractions were collected at 4 °C at a flow rate of 10 mL/h. Buffer A was then run through the column until the OD_{280} returned to baseline, and the column pH was slowly reduced to 3.8 with diluted PB74 (13 mL of PB74 plus 187 mL of distilled water) that had been adjusted to pH 3.8 with 1 mol/L of HCl. Five-milliliter fractions were again collected, this time into tubes containing 0.125 mL of 1 mol/L Tris-Cl (pH 7.5) and 0.10 mL of 1% Tween 20. When the eluate reached pH 3.8,

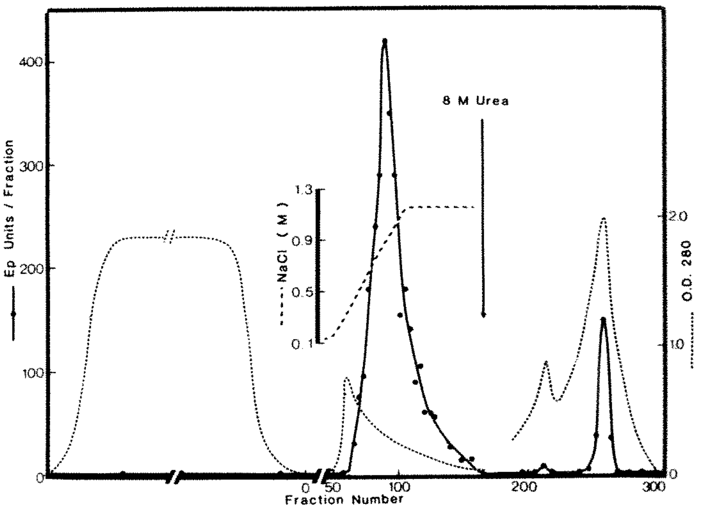

Fig 1. CM Affi-Gel Blue chromatography of filtered human urine. See ref. 5 for complete details.

the column was restored to pH 7.0 with buffer A. To elute most of the Ep activity, 0.3 mol/L of NaCl in buffer A was then applied, and 1.0-mL fractions were collected (into tubes containing 0.02 mL of 1% Tween 20) at 4 °C and at 10 mL/h. Last, 3 mol/L of NaCl in buffer A was put through the column to remove tightly bound proteins and regenerate the gel. A typical profile is shown in Fig 2.

Chromatofocusing was found to be superior to diethylaminoethanol (DEAE) Sephacel chromatography in terms of both column capacity and recovery of Ep activity. Moreover, we found that recoveries were far higher when the pH was returned to 7.0 and when the Ep was eluted with high salt rather than when the pH was either lowered below the isoelectric point of Ep (~3.5) (E. Goldwasser, personal communication) or when eluting was performed with high salt at any pH <4.5. Lowering the pH to 2.9 resulted in the release of only ~50% of the Ep activity; removal of the rest required elution with 0.3 moL/L of NaCl. Although most Ep preparations yielded an elution pattern identical to that shown in Fig 2, approximately one preparation in ten released a substantial amount of total Ep activity (ie, 40% to 50% in some cases) during the initial lowering of the pH to 3.8. This latter elution pattern could be duplicated by treating preparations that gave the standard elution profile with neuraminidase-agarose beads, suggesting that some Ep preparations are less sialated than others, possibly due to patient-to-patient variation in Ep glycosylation or increased losses of sialic acid residues during the purification of some preparations.

Wheat germ Lectin–Sepharose 6MB chromatography. Chromatofocused Ep preparations that displayed the typical elution pattern shown in Fig 2 could be further purified using wheat germ affinity chromatography. The use of wheat germ lectin to purify Ep was first described by Spivak et al,[14] and the conditions used here were modified only slightly from those described by Dukes et al.[15] Specifically, the 0.3 mol/L NaCl pool following chromatofocusing was loaded directly onto a wheat germ Lectin-Sepharose 6MB gel bed (8,000 U/mL of gel) previously equilibrated with 0.02% Tween in PBS. Chromatography was carried out at room temperature and at a flow rate of 2 mL/h. Most of the Ep activity was eluted with 10 mol/L of *N*-acetyl-D-glucosamine in PBS containing 0.02% Tween 20, and the column was then regenerated first with 200 mmol/L of *N*-acetyl-D-glucosamine in the same buffer followed by 20 gel bed vol of 0.02% Tween 20 in PBS. Typically, the bulk of

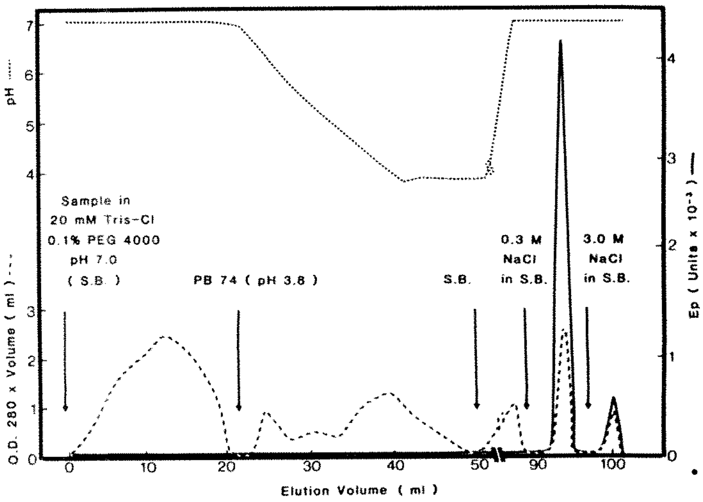

Fig 2. Chromatofocusing of typical step 1 Ep. To monitor the change in pH, every tenth tube did not contain 0.125 mL of 1 mol/L Tris-Cl (pH 2.5). S.B., starting buffer.

the contaminating proteins did not bind to the column, and >90% of the recoverable Ep activity eluted with 10 mmol/L of *N*-acetyl-D-glucosamine (Fig 3). We found that the best purification was achieved if an amount of Ep that closely approached the capacity of the gel was applied, ie, ~8,000 U of chromatofocused Ep per milliliter of gel, using lot No. IF 32137 from Pharmacia Fine Chemicals. However, gel capacity had to be determined through experimentation because it varied markedly from lot to lot. If substantially less than the column capacity of Ep was applied, a significant proportion of the total activity eluted with the 200 mmol/L of *N*-acetyl-D-glucosamine buffer. Using this procedure, we obtained, with most chromatofocused preparations, a fourfold purification with an overall yield of 65%.

Hydroxylapatite chromatography. Approximately one in ten Ep preparations gave an anomalous chromatofocusing profile, consistent with the fact that the Ep in these preparations was less sialated than most of the Ep preparations. The 0.3 mol/L NaCl pools remaining after chromatofocusing of these anomalous preparations were not well suited to wheat germ fractionation, ie, most of the Ep activity did not bind to the lectin. For these preparations, an alternative procedure which involved hydroxylapatite was devised. This chromatographic step was first used by Miyake et al[3] as part of their purification procedure for Ep. However, using their procedure, we achieved extremely low recoveries. This problem could be alleviated somewhat either by applying very high concentrations of protein to the column as Miyake et al[3] did or by adding 0.02% Tween 20 to the chromatographic buffers. This detergent did not appear to interfere with the elution profile. Specific details of the procedure used are given in the legend to Fig 4. Two distinct fractions containing Ep activity were detected, confirming the heterogeneity previously reported with this gel fractionation procedure.[3] When anomalous step 2 Ep was chromatographed on this column, the bulk of the activity eluted with 100 mmol/L of Na/K phosphate (pH 6.8) (Fig 4A), and resulted in a threefold increase in specific activity and a 50% recovery. However, when a typical step 2 Ep preparation was chromatographed, most of the activity eluted between 0 and 10 mmol/L of Na/K phosphate (pH 6.8) (Fig 4B).

Reverse-phase HPLC. Following wheat germ or hydroxylapatite chromatography, the main bulk of Ep activity was concentrated to ~1.5 mL, using an Amicon ultrafiltration unit with a PM 10 membrane, and was loaded onto a 3.9-mm × 30-cm μBondapak phenyl HPLC column equilibrated with 0.1 mol/L of K phosphate (pH 7.0). Samples up to 5 mL, using multiple injections and a 200-μL loop, could be applied with no deleterious effect on subsequent resolution. A biphasic linear 0% to 40% gradient of *n*-propanol in 0.1 mol/L of K phosphate (pH 7.0) was used, and 1-mL fractions were collected into tubes containing a final concentration of either 0.1% SDS or 0.02% Tween 20. Ep eluted as a single peak with ~34% *n*-propanol (Fig 5). Anomalous preparations required slightly more *n*-propanol for elution. This step gave a fivefold increase in purity to ~21,000 U/mg in 70% yield. The active fractions were pooled, dialyzed against 0.01% SDS at 23 °C for six hours, and lyophilized to dryness in preparation for SDS-PAGE.

SDS-PAGE. Ep samples were resuspended in ~2 mL of 62.5 mmol/L of Tris-Cl (pH 6.8), 10% glycerol, and sufficient SDS to give a final concentration of 2%. They were then applied directly to 1.8-mm thick 10% Laemmli gels.[11] The samples were neither heated nor reduced because this diminished subsequent recoveries. Following electrophoresis, 0.2-cm slices above and below the expected Ep region, (assuming an R_f for Ep of ~0.64 compared with the bromphenol blue dye front) were shaken overnight at 4 °C with 0.5 mL of 0.02% Tween 20 in PBS. Centrifuged sample supernatants were assayed for bioactivity as described in the Materials and Methods section, and those fractions containing activity were pooled. Gel slices that contained Ep activity were re-shaken twice with 0.5 mL per slice of 0.02% Tween 20 in PBS, and the three eluates were pooled. Typically, the first extraction released 90% of the recoverable Ep activity, the second released 8%, and the third released 2%. There was total recovery of 85% using this step, and the specific activity was increased ~3.5-fold to 80,000 U/mg.

The five-step purification scheme for Ep from human urine is summarized in Table 1. Overall recovery using this procedure was ~25% as determined by the [^{3}H]-thymidine Ep microassay method. Identical results were obtained using

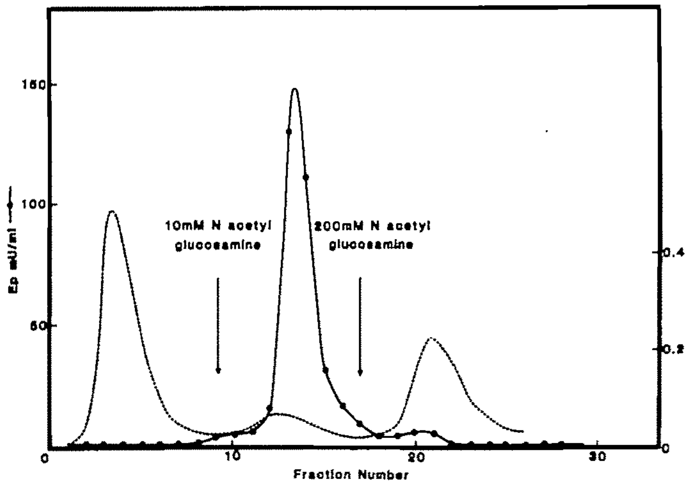

Fig 3. **Wheat germ Lectin–Sepharose 6MB chromatography of typical step 2 Ep.**

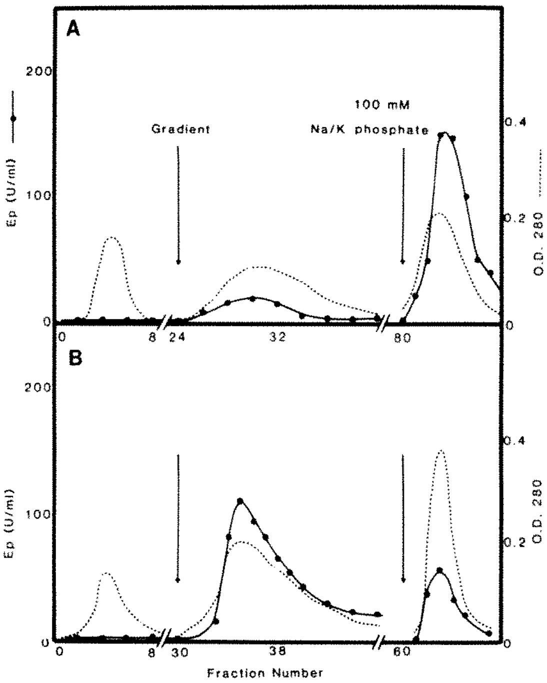

Fig 4. Hydroxylapatite chromatography of (A) anomalous and (B) typical step 2 Ep. One mL of step 2 Ep (1,100 U) was dialyzed against distilled water containing 0.02% Tween 20 and was applied to a 0.5-mL gel bed of hydroxylapatite preequilibrated with distilled water containing 0.02% Tween 20. After administration of the sample, the column was eluted with 0.2% Tween 20 in water, and 1-mL fractions were collected at 4 °C and at 10 mL/h until the OD_{280} returned to baseline. A gradient of 0 to 10 mmol/L of Na/K phosphate was then initiated, using 10 mL of distilled water and 10 mL of 10 mmol/L Na/K phosphate (pH 6.8), both containing 0.02% Tween 20. This was followed by 10 mmol/L of Na/K phosphate until the OD_{280} returned to baseline. Finally, 100 mmol/L of Na/K phosphate (pH 6.8) containing 0.02% Tween 20 was used to elute the remaining activity.

a standard Ep assay based on in vitro ^{59}Fe incorporation.[16] Moreover, the step 5 material was also tested in the in vivo polycythemic mouse assay[8]; the results indicated a potency of 80,000 U/mg, very similar to that obtained with the in vitro assay procedures.

Determination of Purity

Aliquots at each stage of the purification procedure were assessed for purity by analytical SDS-PAGE. For this purpose, samples were reduced with 5% 2-mercaptoethanol and were heated to 100 °C for two minutes. Following electrophoresis, most of the lanes were silver-stained by a modified Ohsawa and Ebata method.[10] The major modification in the silver-staining procedure involved use of 10% glutaraldehyde prior to gel shrinkage with PEG 2000. This was found to make the silver-staining intensity of various test proteins more quantitative, ie, the staining intensity more closely reflected the amount of protein applied to the gels. (G. Krystal and S. Ashwell, unpublished observations) As shown in Fig 6, the final Ep preparation (step 5) appeared as a single broad band on analytical SDS-PAGE; this band coelectrophoresed with Ep bioactivity. Moreover, the average mol wt of this broad band was 38.5 kd, in close agreement with the mol wt assignment reported by Miyake et al.[3] However, the mol wt varied slightly depending on the percentage of acrylamide in the SDS gels. For example, step 5 material appeared to have a mol wt of 39 kd on an 8% gel and 37 kd on a 12% gel. These results are consistent with Ep being a glycosylated protein.[17] One of the anomalous Ep preparations, discussed above, which did not bind to wheat germ and was purified to homogeneity using hydroxylapatite followed by HPLC and preparative SDS-PAGE, appeared to have a mol wt of 32 kd on a 10% acrylamide gel. This is consistent with its being substantially less sialated than most of the Ep preparations which we have purified.

The specific activity of step 5 Ep was estimated by scanning lanes iv and v and comparing their staining intensities with protein standards (Fig 6). This was necessary because the concentration of protein in our eluted step 5 material was too low to determine protein content by more

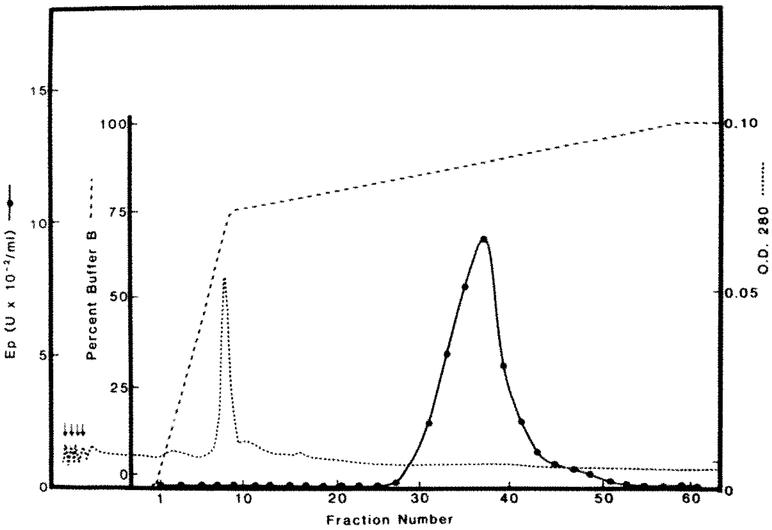

Fig 5. Phenyl high-performance liquid chromatography (HPLC) of step 3 Ep. Approximately 10,120 U (2.4 mg) of step 3 Ep was applied directly, using multiple injections of 200 μL each (arrows indicate the last of these injections), to a 0.39 × 30 cm phenyl HPLC column preequilibrated with 0.1 mol/L of K phosphate (pH 7.0) (buffer A). Chromatography was carried out at 23 °C, at a flow rate of 0.5 mL/min, and 1-mL fractions were collected into tubes containing 10 μL of 10% SDS. For the first five minutes, elution was carried out with buffer A. A biphasic linear gradient was then initiated: at 20 minutes, the percentage of buffer B (40% *n*-propanol in 0.1 mol/L of K phosphate [pH 7.0]) eluting through the column was up to 75%. A shallower gradient was then initiated: at 120 minutes, the column reached 100% buffer B.

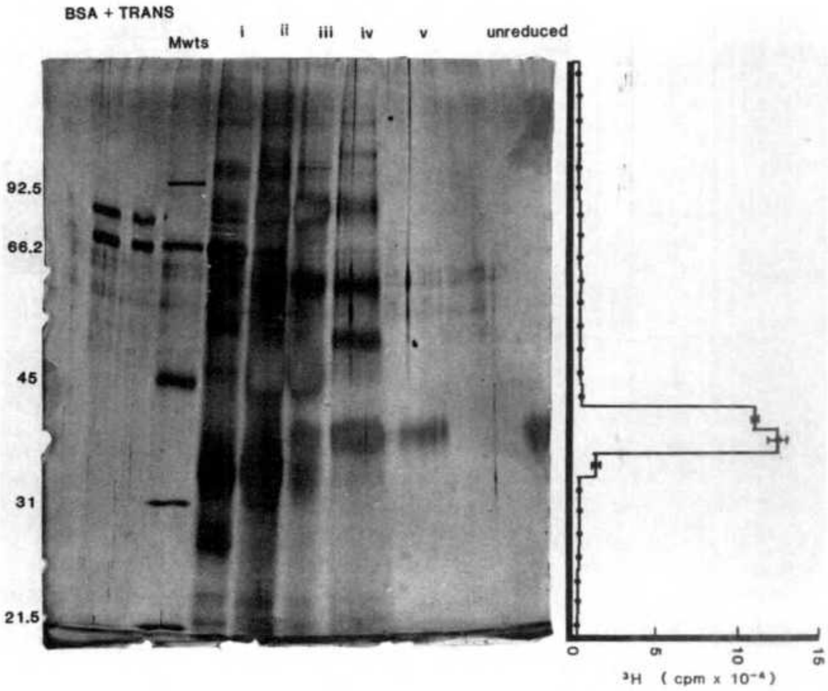

Fig 6. Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) analysis of the Ep fractions from each step in the purification procedure. The gel concentration was 10%. All samples were in 2% SDS, 10% glycerol and 50 mmol/L of Tris-Cl (pH 6.8). Samples applied to lanes 1 through 9 were reduced with 5% 2-mercaptoethanol and were boiled for two minutes. Samples 10 through 12 were neither reduced nor heated. Lanes 1 and 2 contained 100 ng and 50 ng, respectively, of bovine serum albumin and transferrin; lane 3 contained Bio-Rad low mol wt SDS-PAGE standards; lanes 4 through 8 contained Ep of steps 1 through 5, respectively; lane 9 contained reduced and heated SDS sample buffer; lane 10 contained unreduced, unheated SDS sample buffer; lane 11 contained buffer unreduced and unheated step 5 Ep. Lane 12, containing unreduced, unheated step 3 Ep was cut into 0.5-cm slices, shaken overnight in PBS containing 0.02% Tween 20, and bioassayed.

conventional Coomassie,[9] OD_{280},[4] or OD_{278}[3] methods. Scanning of lane iv and measuring of the areas under the peaks demonstrated that 29% of the total silver-staining material was present in the Ep region. Assuming that pure Ep was between 70,000[3] and 80,000[4] U/mg suggested that the HPLC-purified preparation had a sp activity of 20,000 to 23,000 U/mg, consistent with the 21,000 U/mg obtained for this material using Bradford's protein determination method (see Table 1), and lending credence to the scanning of silver-stained gels (using the silver-staining method described above) in order to obtain quantitative data on the protein content of gel bands. Known amounts (50 ng and 100 ng) of BSA and transferrin were electrophoresed on the same gels to help quantitate the amount of protein in lane v (Fig 6); it was determined that, for the same amount of protein, transferrin yielded 75% to 80% of the staining intensity of BSA. Therefore, taking into account band breadth and the fact that 11 U of Ep were applied to lane v, the scanning data suggested that the step 5 Ep preparation was between 69,000 U/mg (based on transferrin) and 89,000 U/mg (based on BSA). This is in close agreement with the previously reported specific activities for pure Ep of 66 U/mg,[18] 70.4 U/mg,[3] and 81.6 U/mg.[4]

Close examination of lanes 1 through 9 in Fig 6 revealed three faint bands between 55 kd and 62 kd. Because only SDS sample buffer with 5% mercaptoethanol was run in lane 9, it was concluded that these proteins were contaminants from the SDS sample buffer and were not present in the Ep preparations themselves. Moreover, these bands were observed when SDS sample buffers containing either mercaptoethanol (lane 9, Fig 6) or dithiothreitol (data not shown) were used but not when SDS sample buffers without reducing agents were used (lanes 10 and 11, Fig 6). These findings are in agreement with previous reports[19,20] which suggest that these contaminants are of human skin origin and that they are far more readily observed in the presence of reducing agents.[20]

Reduced electrophoretically pure Ep migrated marginally slower than did unreduced Ep (lane 12, Fig 6). This is consistent with the presence of an intrachain disulfide bridge in the native molecule, and is in agreement with a recent report by Jacobs et al in which they suggest that at least two of the four cysteines in Ep may be involved in a disulfide bond.[21] It was also noted that unreduced Ep appeared on our SDS gels as a slightly more diffuse band than that of reduced Ep. This may suggest that not all Ep molecules have the same number of disulfide bridges in the native state.

The purity of step 5 Ep was firmly established by amino acid sequencing. The material was subjected to 18 cycles of NH_2-terminal amino acid sequence analysis as described in the Materials and Methods section. Approximately 200 pmols of protein was analyzed by sequential stepwise Edman degradation yielding the following unique polypeptide sequence: ?-?-Pro-Arg-Leu-Ile-?-Asp-Ser-?-Val-Leu-Glu-?-Tyr-Leu-Leu-Glu. The first two amino acids were not determinable because of contamination of the step 5 Ep with glycine and Tween 20 from the electrophoresis step. The sequence obtained is in total agreement with three previously reported Ep sequences.[4,21,22] Consequently, we can explain the lack of scores at positions 10 and 14 by noting that the previously cited Ep sequences predict Arg residues at these positions, which give very low PTH yields and would be below the level of detection in our sample.

Biochemical Characterization of Step 5 Ep

Step 5 Ep was iodinated using [^{125}I]-NaI and chloramine T[12] and was then subjected to trypsin digestion as described in the Materials and Methods section. Reverse-phase HPLC

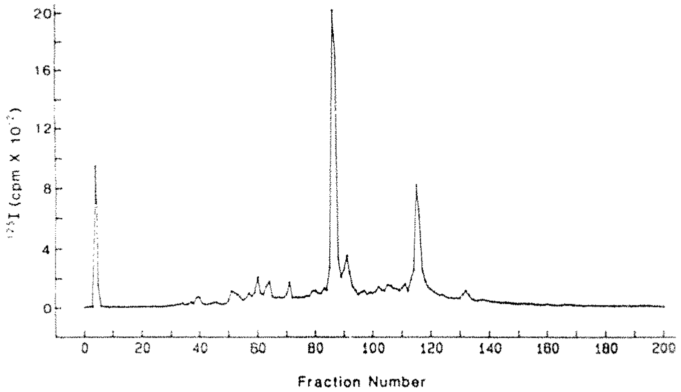

Fig 7. C_{18} high-performance liquid chromatography of trypsinized step 5 Ep.

using a C_{18} column and a linear gradient of ethanol from 0% to 62.5% revealed two well-defined peaks (Fig 7). This profile has been obtained by Smart and Farber (J.E. Smart and N.M. Farber, unpublished observations) with several homogeneous preparations of Ep, including one that they have sequenced extensively (48%) and that shows 100% agreement with the recently published sequence for Ep.[21]

DISCUSSION

In this report, a method for the purification of human urinary Ep to apparent homogeneity is described. The advantages of this method over that previously presented by Miyake et al[3] are: first, fewer steps are used; second, far less starting urine is required to ensure reasonable final recoveries; and third, significantly higher final recoveries (25% *v* 15%) are obtainable. Thus, investigators who have been anxious for homogeneous preparations of Ep but who have been hindered by limited supplies of high Ep titer urine can now obtain ~1,250 U of pure Ep from only 5,000 U of starting urine.

The first two steps in this new procedure, namely CM Affi-Gel Blue chromatography and chromatofocusing, serve not only to purify but to concentrate as well. Thus, these two high-protein-capacity gels are well suited for the first two fractionation steps. The last four steps, ie, chromatofocusing, wheat germ lectin–Sepharose 6MB chromatography, reverse-phase HPLC and SDS-PAGE, all provide information concerning the amount of heterogeneity in the particular Ep preparation being processed. For example, both the anomalous Ep preparations and neuraminidase-treated typical Ep preparations displayed: (1) an increased Ep activity in the unbound flow through and the PB74 fractions following chromatofocusing, (2) less binding to wheat germ lectin beads, (3) a greater proportion of the eluted Ep activity, following hydroxylapatite, in the 100 mmol/L Na/K phosphate pool, and (4) an increased binding affinity to the phenyl-HPLC column, ie, such that elution required higher concentrations of *n*-propanol. This last phenomenon is compatible with these Ep preparations being less sialated, since removal of negative sialic acid residues would be expected to make Ep more hydrophobic and would thus require a more hydrophobic buffer for elution. Finally, some anomalous Ep preparations and neuraminidase-treated typical Ep preparations displayed mol wt as low as 32 kd, significantly lower than the 38.5 kd seen with untreated typical Ep preparations. This dramatic difference in mol wt is not inconsistent with simple desialation of native Ep, since Goldwasser has reported that there are between 16 and 18 sialic acid residues per molecule of native Ep.[23] This ability to assess the degree of sialation during Ep purification is a great asset since it is well established that desialated Ep is without effect in vivo.[24] Thus, for example, researchers who are interested in purifying Ep for therapeutic administration to patients can abort their purification procedure at the chromatofocusing stage if anomalous elution profiles are observed. This feature may also be of more general interest since desialated Ep has been reported to be less stable than the native hormone.[24]

Trypsinized pure ^{125}I-labeled Ep yields two well-defined peaks of radioactivity upon Spherisorb 10 ODS (C_{18}) reverse-phase HPLC. This finding can therefore be used as a fast check of Ep purity since it requires far less time and material (ie, 100-fold less) than does gas-phase amino acid sequencing.

The recently published cDNA nucleotide (and therefore amino acid) sequence for Ep[21] suggests that approximately one-half of the Ep molecule must be contributed by sugar side chains. The availability of both recombinant and human urine-derived Ep should now permit a more detailed examination of the contribution by these sugar moieties. It will also be of value to determine whether recombinant Ep that is glycosylated in non-human cells has a shorter half-life in the human circulatory system than does human urine-derived Ep.

ACKNOWLEDGMENT

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Detection of Clonal Excess in Lymphoproliferative Disease by κ/λ Analysis: Correlation With Immunoglobulin Gene DNA Rearrangement

By Nancy Berliner, Kenneth A. Ault, Paula Martin, and David S. Weinberg

Previous studies have suggested that analysis of the distribution of surface immunoglobulin light chain isotypes by flow cytometry provides evidence for monoclonality of B cell tumors and may detect populations of circulating tumor cells in patients with lymphoproliferative disease. We have used simultaneous flow cytometry and DNA restriction enzyme analysis on 58 samples of tissue and blood to determine whether lymphocyte populations detected by "κ/λ" analysis are indeed monoclonal. In >90% of cases, abnormalities detected by flow cytometry correlated with monoclonal rearrangements of immunoglobulin genes as detected by Southern blot analysis. By analyzing tissue and blood from the same patients, we have also demonstrated that monoclonal circulating cells detected by flow cytometry reflect peripheral circulating tumor cells, since DNA from these cells shows the same immunoglobulin rearrangement as DNA from the original tumors in these patients. Although mixing studies suggested that DNA rearrangement studies were more sensitive than was flow cytometry in detecting minor populations of monoclonal lymphocytes, we found only one case in which this affected the diagnostic accuracy of the κ/λ analysis, with one notable exception, that of detection of a monoclonal proliferation of B cells that did not express surface immunoglobulin. The κ/λ test thus offers a powerful diagnostic tool in the evaluation of lymphoproliferative disease.

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THE EVOLVING understanding of the ontogeny of the B lymphocyte has resulted in the development of immunologic methods that have had a great impact on the diagnosis of lymphoproliferative disease. Most malignant lymphomas are of B cell origin.[1,2] Most B cells, with the exception of very early pre-B cells and mature plasma cells, express immunoglobulin on their surface.[3] Each cell expresses only one light chain type. In normal peripheral blood and lymph nodes, there is usually a mixture of κ-positive and λ-positive cells, with two-thirds of the cells expressing κ and one-third expressing λ. Because lymphoid neoplasms are usually clonal expansions of a single cell, the cells uniformly express the same light chain isotype. Consequently, B cell malignancy can frequently be suspected on the basis of the demonstration of a marked predominance of cells expressing a single light chain type.[4]

The uniform expression of immunoglobulin light chains on monoclonal cell populations has been exploited in the development of a cytofluorometric technique to detect small populations of monoclonal B lymphocytes. This κ/λ technique is based on the direct comparison of surface immunofluorescent staining for κ and λ light chains within a cell population. Evidence of an imbalance in the normal distribution of light chain staining implies the presence of a monoclonal cell population. This technique has been used to show that a large proportion of patients with B cell lymphomas have circulating monoclonal B cells that are morphologically undetectable.[5] κ/λ analysis has also been used to distinguish lymphoid neoplasms from benign reactive processes.[6]

Although it has been assumed that an abnormal light chain fluorescence distribution indicates a monoclonal B cell proliferation, direct proof of this has been lacking. The availability of recombinant DNA techniques has made it possible to confirm that assumption. Immunoglobulin molecules are encoded on discontinuous genes. Early in B cell development, the immunoglobulin loci undergo gene rearrangement to form functional coding units for antibody synthesis. This rearrangement is detectable on analysis of genomic DNA and may provide evidence of monoclonality of B cells at very early stages of B cell development.[7,8] This powerful tool has been used to establish the monoclonality of B cell neoplasms in which the diagnosis is hindered by confusing histology or absence of surface markers and has recently been used to establish that some lymphoid malignancies may in fact consist of more than one clone.[9,10]

In this study, we have used DNA restriction enzyme analysis to assess the validity of the κ/λ test. We have confirmed that the detection of clonal excess by flow cytometry correlates well with the finding of monoclonal immunoglobulin rearrangements by DNA analysis. We have also shown that the detection by κ/λ analysis of small populations of tumor cells in the peripheral blood of patients with lymphoma reflects a population of circulating cells with the same DNA rearrangement as that seen in the primary tumors in those patients. We have identified the circumstances under which DNA restriction enzyme analysis is more informative than flow cytometry in the analysis of small populations of B cells. We conclude that the κ/λ test offers a useful and easily applied method for the diagnosis and analysis of lymphoid malignancy.

MATERIALS AND METHODS

Patient selection. Fifty-eight samples of tissue and peripheral blood from patients at the Brigham and Women's Hospital who had proven or suspected lymphoproliferative diseases were obtained for analysis. Diagnoses are outlined in Table 1. The non-Hodgkin's lymphomas included a broad range of histologic types. Because the

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Table 1. Summary of Patients Studied

Diagnosis	No. of Patients	
	Tissue	Blood
Chronic lymphocytic leukemia (B cell)	1	8
Hairy cell leukemia	1	3
Non-Hodgkin's lymphoma	18*	17†
Reactive	6	2
Lymphosarcoma cell leukemia	0	1
Hodgkin's disease	1	0

*Includes 15 B cell type, 1 T cell, 1 null cell, and 1 true histiocytic cell by immunoperoxidase studies.

†All patients had non-Hodgkin's lymphomas of demonstrated B cell type.

study was intended to compare the capabilities of κ/λ and Southern blot analysis in detecting clonal B cell proliferations, we were concerned mainly with the phenotypes of the various lymphomas. Each diagnosis was confirmed by routine histology, and the phenotype was established by immunoperoxidase staining of cryostat sections.[6] All studies were performed with the approval of the BWH human subjects committee.

κ/λ analysis. Lymphocytes were obtained by Ficoll-Hypaque centrifugation of peripheral blood or cell suspensions of tissue samples. The cells were separated into three aliquots. One was reserved for DNA analysis. The other two aliquots were labeled with rabbit heteroantisera to immunoglobulin light chains, one with κ and the other with λ. The staining procedure has been described previously.[6] Cells (1 to 2 × 10^4) from each sample were analyzed for fluorescence on a flow cytometer, and histograms were obtained by plotting relative cell number *v* log fluorescence intensity. The two curves obtained in this manner were analyzed for evidence of a monoclonal population. The studies were performed on a FACS Analyzer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif). This instrument measures electronic volume and single-cell fluorescence excited by filtered light from a mercury-arc lamp.

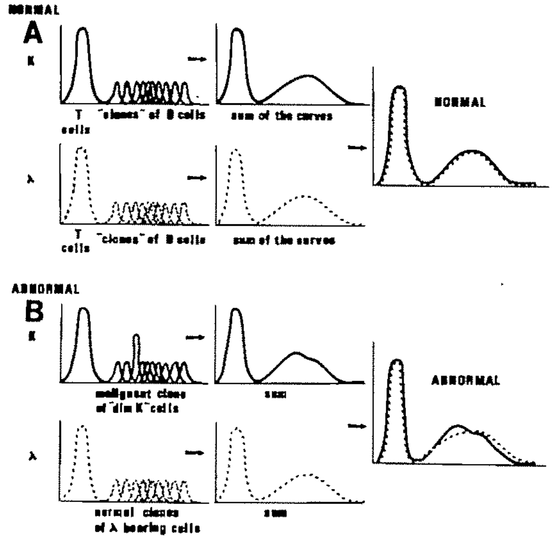

Fig 1. Detection of monoclonal B lymphocytes by "kappa-lambda" analysis; basis of the κ/λ test. Idealized fluorescence distribution curves are depicted for cells stained for surface light chains and analyzed by flow cytometry. Increasing log fluorescence intensity is shown on the horizontal axis; cell number is represented on the vertical axis. As noted in the text, each clone of B cells expresses either κ or λ light chains, but not both. The fluorescence distribution profile for each light chain is determined separately. As demonstrated here, the fluorescence distribution profile for each light chain represents a summation of the fluorescent intensity of each individual B cell clone. In normal individuals, the frequency distribution for the two light chain types is nearly identical. As shown in (A), the relative shapes and positions of the two curves are the same for both light chains. The effect of an abnormal clone of κ/λ expressing cells on the fluorescence distribution curves is demonstrated in (B). The κ distribution curve is shifted, whereas the λ light chain curve is unchanged. The difference is most obvious when the two curves are superimposed; this difference may be quantitated by computer analysis.

The basis for interpretation of histograms obtained by κ/λ analysis is outlined in Fig 1 (similar to that previously published; see ref. 6). Detection of clonal excess depends on the empiric observation that normal κ and λ histograms are identical in shape. Each curve shows a frequency distribution of fluorescence around a mean fluorescent intensity. This mean fluorescence intensity is identical for both κ-staining and λ-staining cells, and is independent of the relative numbers of cells that are positive for each light chain. As noted above, κ cells normally outnumber λ cells by a ratio of ~2:1. However, when the two curves are normalized for cell number, they become superimposable as shown in the upper panel (Fig 1). The presence of an abnormal clone (or clones) is detectable by the presence of a shift in the fluorescence distribution curves as shown in the lower panel (Fig 1). The proliferation of a clone of cells with a uniform fluorescence intensity of a single light chain causes a shift of that curve relative to the other unaffected light chain; as a result, the two curves are no longer superimposable. Differences are usually obvious on inspection of the curves. Curves were also analyzed by computer, using the Kolmogorov–Smirnov test[11] to derive a "D" value to express the differences between them. This value reflects the magnitude of the deviation of the two curves and is expressed as a percentage of the total number of cells analyzed.[6] It is independent of the number of cells staining for each light chain and provides a quantitative comparison of the two fluorescence distributions. Previous studies in this laboratory have shown that the range of D values seen in the evaluation of blood from normal individuals is 5.1 ± 3.3 (± 2 SD). A D value of 10 is thus considered to be the upper limit of normal. Occasional false-positive results have been observed in individuals with nonlymphoproliferative disorders and, rarely, in normal individuals. Consequently, studies must be correlated with appropriate clinical and histologic information.

Flow cytometry also allows fluorescence analysis of cells within selected size ranges. In some cases, cells were divided into "small" and "large" cells by selecting appropriate cell volume gates; each group was then analyzed separately for light chain distribution.[6]

DNA rearrangement analysis. High mol wt DNA was prepared from Ficoll-Hypaque gradient-purified mononuclear cells obtained as noted above. Most samples contained between 5 × 10^6 and 10^7 cells. Cells were lysed with 1% Triton X-100 in 32% sucrose at 4 °C to obtain nuclei. Nuclei were incubated overnight at 37 °C in 0.5% SDS and 0.2 mg/mL of Proteinase K. This was followed by phenol-chloroform extraction and ethanol precipitation as described by Bell et al.[12] DNA was digested with appropriate restriction enzymes, size-fractionated by agarose gel electrophoresis and transferred onto nitrocellulose paper by the method of Southern.[13] Filters were hybridized to nick-translated, ^{32}P-labeled probes of the immunoglobulin κ and λ light chain loci and were washed at 53 °C in 0.1% SDS/0.15 mol/L of NaCl–0.00 15 mol/L of Na citrate prior to autoradiography.

The probes used for Southern analysis are shown in Fig 2. The appropriate restriction sites for each probe are shown. V-J recombination in the light chain loci gives rise to an altered 5′ restriction

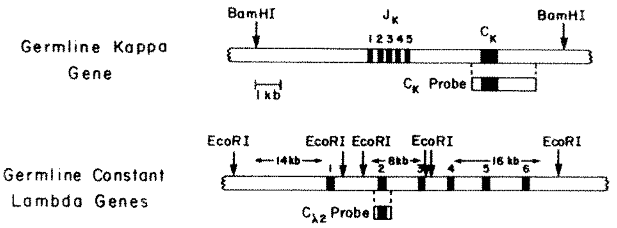

Fig 2. Probes used for DNA rearrangement studies. The restriction sites for enzymatic digestion within the light chain loci are shown. κ gene rearrangement is most easily seen using a probe derived from the κ-constant region to analyze DNA digested with Bam HI. This yields a single 12-kilobase (kb) fragment in germline DNA. λ rearrangements were demonstrated by analysis of Eco RI-digested DNA with a probe from the second constant region of the λ locus. This probe is derived from an area of Cλ2 that is highly homologous to the other constant region genes. Consequently, analysis of germline DNA typically reveals three bands of 8, 14, and 16 kb. However, the region of Cλ2 is polymorphic,[14] and the 8-kb band may be 13, 18, or, infrequently, 23 kb in length. This occasionally may make analysis of rearranged bands confusing; in such cases, granulocytes were used as a source of germline DNA for comparison and confirmation of rearrangements. The Cλ2 probe also hybridizes faintly to a 5-kb fragment that represents a pseudogene.

enzyme site and alters the size of the germline restriction fragment. Detection of one or two bands of altered size is indicative of monoclonal rearrangement of the light chain genes. Polyclonal proliferations yield small clones of varied size and do not appear as distinct bands on Southern blot hybridization. All DNAs were also probed with a probe derived from the heavy chain J region (not shown). Because heavy chain rearrangement occurs before light chain rearrangement,[8] all DNAs which displayed light chain rearrangement had also undergone alterations in their heavy chain locus. No DNAs analyzed had a heavy chain rearrangement in the absence of light chain rearrangement.

The number of λ constant region genes has been shown to vary from individual to individual.[14] Consequently, the 8-kilobase (kb) fragment diagrammed in Fig 2 may be 13, 16, or 18 kb. Because this locus has a polymorphic nature, granulocytes from patients with confusing λ gene rearrangements were run as controls. Such polymorphism does not exist in the κ locus; therefore, similar comparisons were unnecessary.

RESULTS

Detection of lymphoma cells by κ/λ and DNA restriction analysis. The ability to detect clonal excess by flow cytometry, as reflected in the D value, is correlated with the findings on DNA analysis in Fig 3. A κ/λ shift (indicated by a D value >10) was detected in 32 of 35 (92%) of cases in which monoclonal immunoglobulin rearrangements were seen on DNA analysis. The three false-negative κ/λ studies, as well as the single case in which a positive κ/λ study was not corroborated by gene rearrangement studies, will be discussed in detail below.

Characteristic results of flow cytometry and DNA rearrangement studies are compared in Fig 4. Panel A shows results obtained by each method in analysis of a lymph node involved by a κ-expressing lymphoma. There is a clearly detectable κ shift by flow cytometry, which correlates with an obvious rearrangement of the κ light chain locus seen on Southern blot analysis. The abnormal result in Fig 4A is compared with the analysis of a benign hyperplastic lymph node, in which the κ/λ curves are superimposable (D < 10), and there is no evidence of a clonal gene rearrangement.

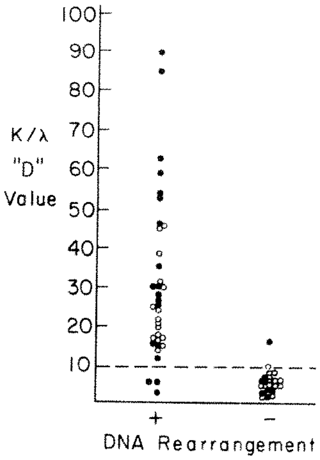

Fig 3. Comparison of results obtained by κ/λ and DNA analysis; κ/λ results, represented by the D value, are compared with the finding of the presence (+) or absence (−) of immunoglobulin gene rearrangement by restriction enzyme analysis. Using the DNA analysis as the standard, three false-negative results and one false-positive result were obtained by cytofluorometric analysis. Tissue, ●; blood, ○.

Lymphoid tissue usually contains cells of variable size. As noted above, flow cytometry allows the separate analysis of cell subpopulations of different size within a total cell population. Previous studies have shown that analyzing cells in this way allows detection of a clonal excess which may be seen only in one such subpopulation.[6] Figure 4B illustrates such a case. By flow cytometry, a clear λ shift is seen only in the large cell population, which constituted a minority of the suspended cells. This correlated with a λ locus rearrangement which was easily detected on DNA restriction fragment analysis of unsorted cells.

Correlation of blood abnormalities with tissue involvement. Tissue and blood from the same patient were obtained in ten cases as outlined in Table 2. Although only one of the 10 showed morphological evidence of peripheral blood involvement with lymphoma, κ/λ and DNA analyses were positive for lymphoma cells in four cases. All four showed identical DNA rearrangement studies in tissue and blood, confirming that shifts seen by flow cytometry correlated with the presence of circulating tumor cells.

Results of blood and tissue studies performed on one such patient are shown in Fig 4C. Flow cytometry reveals a clonal excess of λ-bearing cells in both tissue and blood. DNA analysis confirms that the clone detected in peripheral blood contains a rearrangement of the λ locus identical to that seen in the original tissue specimen.

Discrepancies between flow cytometry and DNA analysis. Previous studies have shown that 10% monoclonal B cells can be routinely detected by flow cytometry.[5] The relative sensitivities of the flow cytometric method and Southern blot analysis were directly compared in mixing studies. Varying numbers of cells from the DAUDI cell line•

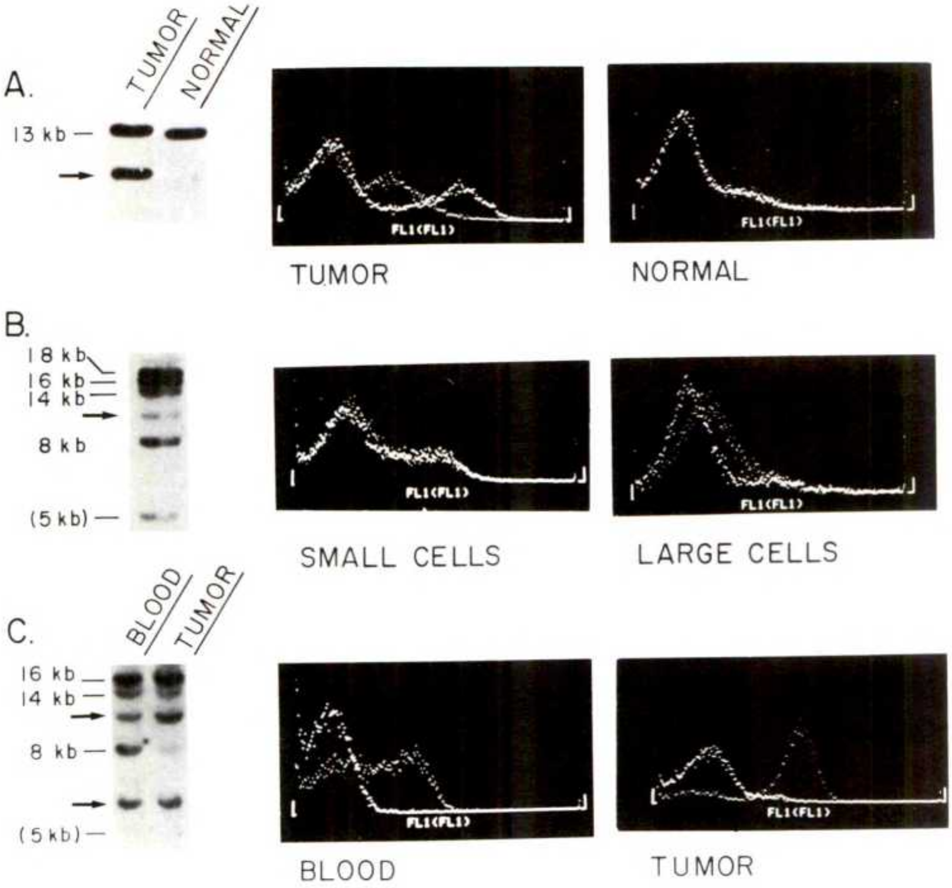

Fig 4. Characteristic DNA and κ/λ analyses. Representative examples of tissue and blood analyses are shown. (A) Typical rearrangement of the kappa light chain locus in lymphocytes from a lymph node involved with lymphoma, with the corresponding abnormal study by flow cytometry (κ, bright dots; λ, dim dots; $D = 53.7$). This study is compared with that of a benign reactive lymph node which shows no light chain abnormality by κ/λ analysis ($D < 10$) and germline κ chain genes with no clonal rearrangement. (B) Analysis of a lymph node with a smaller population of abnormal cells; κ/λ analysis was performed by selective analysis of cells by size. An abnormal clone was detected only in the large cell population ($D = 25$); the corresponding λ light chain rearrangement is seen on restriction enzyme analysis of DNA. Note the presence of the 5-kilobase (kb) fragment which, as noted above, represents hybridization to a known pseudogene. (C) Comparison of blood and tissue specimens from a patient with B cell non-Hodgkin's lymphoma (case 9, Table 2). DNA analysis shows two rearranged bands in the λ locus which are seen on analysis of both blood and tissue. The 8-kb band is very faint in the tumor DNA, consistent with rearrangement of $C\lambda 2$ involving nearly every cell. The germline band is easily seen in the blood analysis, because only a portion of the cells are tumor cells and contain the rearrangement. The DNA studies are compared with the results by flow cytometry. Again, the cells from the tumor show a single population of nearly uniform fluorescence intensity ($D = 52.4$), whereas this is seen as only a part of the cell population in peripheral blood ($D = 46.1$).

(a monoclonal B cell line that exhibits bright surface κ-staining) were mixed with normal spleen cells, and aliquots were subjected to both κ/λ and Southern blot analysis. The lowest proportion of monoclonal B cells detected by κ/λ analysis (ie, $D > 10\%$) was 5%, whereas a distinct band could still be detected on Southern blots with 1% malignant cells. This level of sensitivity of Southern blot analysis is similar to results reported by others.[11] The level of sensitivity of the κ/λ study is in fact somewhat better than previously reported; this is probably owing to the fact that the DAUDI cells exhibit particularly bright staining. Therefore, Southern blot analysis is approximately five to ten times more sensitive than κ/λ analysis in detecting monoclonal B cells. Despite this increase in sensitivity, DNA studies detected no lymphoma cells in peripheral blood samples that were not detected by the κ/λ test. The overall incidence of involvement of peripheral blood with lymphoma cells was 50% by both methods.

In only one of the three false-negative κ/λ tests on tissue samples was the sensitivity of the DNA analysis the probable reason for detecting the clonal cell population. The other two cases involved lymphoma cells with no detectable surface immunoglobulin; in both cases, the monoclonal population was well in excess of 10% of the total cells. The one false-positive case occurred in a patient diagnosed as having true histiocytic lymphoma. This diagnosis was based on immunoperoxidase and histocytochemical studies which showed that the large malignant cells lacked T cell-related or B cell-related antigens or immunoglobulin light chains, but were positive for monocyte/macrophage-related antigen (MO-2), intracellular lysozyme, and α-naphthyl acetate esterase activity.

Table 2. Comparison of Tissue and Blood From Ten Patients

Case	Diagnosis	Tissue κ/λ	Tissue DNA	Blood κ/λ	Blood DNA
1	ML-H (N + D)	λ	λ	—	—
2	ILL	κ	κ	—	—
3	DUL	κ	κ	—	—
4	PDL (N + D)	λ	λ	—	—
5	PDL (N + D)	λ	λ	—	—
6	ILL	λ	λ	λ	λ
7	ML-H (D)	κ	κ	—	—
8	CLL	κ	κ	κ	κ
9	ILL	λ	λ	λ	λ
10	CLL	λ	λ	λ	λ

ML-H, mixed lymphocytic-histiocytic lymphoma; ILL, intermediate lymphocytic lymphoma; DUL, diffuse undifferentiated lymphoma; PDL, poorly differentiated lymphocytic lymphoma; CLL, chronic lymphocytic leukemia; N, nodular; D, diffuse. All diagnoses refer to Rappaport classification, as modified to include ILL.[15]

The misassignment of light chain type to a small clone of abnormal cells is a potential error of the κ/λ test. A given clone is detectable because it has a uniform fluorescence. This fluorescence may be either brighter or dimmer than the mean fluorescence of a population of normal cells. Consequently, when the distortion of the curve is small, it is often difficult to ascertain whether the clone is of one light chain or another, although there may clearly be a clone present. This is, of course, not a problem with DNA analysis. In this study, only one sample was assigned an inappropriate light chain type by flow cytometry.

DISCUSSION

By the use of DNA restriction enzyme analysis, we have confirmed that the light chain imbalance detected by the κ/λ test is reflective of a monoclonal cell population in patients with non-Hodgkin's lymphoma. Further, we have documented that clonal excess detected in peripheral blood by flow cytometry correlates with the presence of circulating lymphoma cells identical to those present in tumor tissue.

The κ/λ test appears to be a very sensitive means of assessing cell populations for the presence of abnormal clones of B cells. In an analytic cytology lab equipped with a flow cytometer, it offers significant advantages over DNA analysis. It is less time-consuming and does not require radiolabeled probes. The κ/λ test may also be done on as few as 10^4 cells, whereas DNA studies usually require 5×10^6 cells.

In this study, we have tried to establish the circumstances under which DNA analysis offers significant advantages over κ/λ studies. There are two major reasons for false negative-results in flow cytometry analysis: technical and biologic. The studies may be inaccurate for technical reasons on several grounds: (1) the size of the clonal population may be below the limits of detectability of the κ/λ test; (2) the curves may be difficult to interpret and may lead to assignment of the incorrect light chain type; or (3) the test may be technically inadequate. In this study, few errors in diagnosis were made as a result of technique. In only one sample of the total 58 was the abnormal population detectable by DNA analysis so small as to be undetectable by flow cytometry; in one case, the light chain type was incorrectly assigned. Hence, although DNA restriction analysis is more sensitive than κ/λ analysis, it had little effect on the diagnostic accuracy of the test.

False-negative results can also arise for biologic reasons. The κ/λ test cannot detect abnormal lymphoid populations if the cells lack surface immunoglobulin, as was true in two cases studied here. In such cases, DNA analysis obviously offers significant advantages in detecting abnormal cell populations, as has been shown by others.[9]

We observed one positive κ/λ study that was not correlated with detectable DNA rearrangements. The reason for this apparent false-positive study is unclear, but there are several possibilities. Either study may have been technically at fault, although this would be surprising with a D value of 16, which suggests a significant clonal population that should be unmistakable by κ/λ analysis and easily detectable by DNA studies. Alternatively, it may reflect the biologic nature of the tumor involved. This case was diagnosed as a true histiocytic lymphoma, based on generally accepted histologic, immunologic, and histocytochemical criteria.[15] The malignant cells in this disorder phenotypically resemble normal monocytes or macrophages, and may be associated with Fc receptors with high avidity for circulating immunoglobulin.[16,17] As in this case, one would anticipate a greater nonspecific binding of κ chains than λ chains, since κ immunoglobulin is present at approximately twice the concentration of λ immunoglobulin in peripheral blood. Although cells are routinely incubated at 37 °C before staining for κ/λ analysis in order to shed cytophilic antibody, this may not have been sufficient in the presence of an avid Fc receptor. This is a potential problem with the κ/λ test, and one that has been observed in patients with M components as is seen in multiple myeloma. It is not usually seen in the presence of normal levels of immunoglobulin, presumably because the nonspecific affinity of lymphocytes for antibody is low; however, it may explain false-positive results seen in patients with high levels of circulating immunoglobulin.

In the current study, we found that 50% of the patients overall with non-Hodgkin's lymphoma of B cell type had evidence of peripheral blood involvement. This figure is somewhat less than the 80% incidence detected by κ/λ analysis recently reported.[18] There are several possible explanations for the lower rate of involvement seen in the current study. First, fewer patients were included in the present study, and inclusion of more patients may have significantly altered the proportion of positive studies. Second, the prior study included many patients with long-standing or relapsed lymphoma, whereas the present study primarily included patients at or near the time of first diagnosis. The fact that circulating lymphoma cells are present in most patients with relapsed lymphoma[18] could explain the greater incidence of positive peripheral blood findings in the earlier study.

In addition to confirming the diagnostic usefulness of the κ/λ test, our results suggest an unexpected aspect of the natural history of lymphoproliferative disease. One might have expected that use of a more sensitive test would have revealed additional cases of peripheral blood involvement undetected by κ/λ analysis. This was not what we observed, however. There were no false-negative κ/λ tests on peripheral blood as compared with DNA rearrangement studies, suggesting that the ability to invade the peripheral blood may be an intrinsic property of certain lymphomas but not of others, despite their apparent similarity by histologic examination. We did not select patients on the basis of the duration of their disease or on their exposure to chemotherapy, both of which may be crucial to the observation of peripheral blood involvement. Whether those patients showing no peripheral blood involvement later develop evidence of circulating clonal cells and whether it becomes detectable by DNA rearrangements before it is detectable by κ/λ analysis must be studied. Appropriate studies of patients with lymphoproliferative disease followed in a longitudinal fashion should aid in elucidating this further.

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Platelet Turnover and Kinetics in Immune Thrombocytopenic Purpura: Results With Autologous ^{111}In-Labeled Platelets and Homologous ^{51}Cr-Labeled Platelets Differ

By Anthon du P. Heyns, Philip N. Badenhorst, Mattheus G. Lötter, Henry Pieters, Paula Wessels, and Harry F. Kotzé

Mean platelet survival and turnover were simultaneously determined with autologous ^{111}In-labeled platelets (^{111}In-AP) and homologous ^{51}Cr-labeled platelets (^{51}Cr-HP) in ten patients with chronic immune thrombocytopenic purpura (ITP). In vivo redistribution of the ^{111}In-AP was quantitated with a scintillation camera and computer-assisted image analysis. The patients were divided into two groups: those with splenic platelet sequestration (spleen-liver ^{111}In activity ratio >1.4), and those with diffuse sequestration in the reticuloendothelial system. The latter patients had more severe ITP reflected by pronounced thrombocytopenia, decreased platelet turnover, and prominent early hepatic platelet sequestration. Mean platelet life span estimated with ^{51}Cr-HP was consistently shorter than that of ^{111}In-AP. Platelet turnover determined with ^{51}Cr-HP was thus overestimated. The difference in results with the two isotope labels was apparently due to greater in vivo elution of ^{51}Cr. Although the limitations of the techniques should be taken into account, these findings indicate that platelet turnover is not always normal or increased in ITP, but is low in severe disease. We suggest that this may be ascribed to damage to megakaryocytes by antiplatelet antibody. The physical characteristics in ^{111}In clearly make this radionuclide superior to ^{51}Cr for the study of platelet kinetics in ITP.

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PLATELET turnover is generally regarded as normal or increased in chronic immune thrombocytopenic purpura (ITP).[1-4] However, the estimate of the extent of the increase in platelet turnover varies from a mean of 4.9 times normal[1] to 2.3 times normal.[2] This difference has been ascribed to difficulties to accurately estimate of platelet life span in ITP.[5] Because the calculation of the platelet turnover is dependent on the estimate of the platelet life span, it is clear that the result of the latter is critical.

The ^{51}Cr labeling technique[6] is used in most laboratories for the determination of platelet survival. This radionuclide has a low binding efficiency for platelets and, in ITP with severe thrombocytopenia, it is impossible to determine the life span of autologous labeled platelets by this method. ^{111}In-oxine is a superior platelet label,[7] and its high gamma photon yield facilitates quantitative imaging of the in vivo distribution of labeled platelets.[8] ^{111}In labels platelets with high efficiency, permitting kinetic studies with autologous platelets even in the presence of severe thrombocytopenia.[9,10]

We have further investigated our previous finding that platelet turnover is not always increased in ITP[9] and now relate this to the pattern of platelet sequestration in the disease. We compared the results of estimates of platelet life span with ^{111}In-oxine-labeled autologous platelets (^{111}In-AP) with that determined by ^{51}Cr-labeled homologous platelets (^{51}Cr-HP). The results differed; this influenced the estimates of platelet turnover. Turnover estimated with ^{111}In-AP was usually increased in those patients with a splenic sequestration pattern, but was decreased in patients in whom ^{111}In-AP were destroyed diffusely in the reticuloendothelial system (RES). In ITP it seems that the ^{51}Cr labeling method spuriously reduces the estimate of mean platelet survival time, and platelet turnover is therefore erroneously overestimated.

MATERIALS AND METHODS

Patients

Ten patients (patients 1 to 10) with ITP, and not on any treatment, gave informed, written consent to participate in a study approved by the Ethical Committee of the University of the Orange Free State. ITP was diagnosed on the basis of conventional clinical criteria: adult-onset thrombocytopenia of undetermined etiology, normal or increased numbers of bone marrow magakaryocytes, and the absence of a demonstrable cause of platelet destruction or utilization such as systemic lupus erythematosus, drug ingestion, or hypersplenism. Relevant patient details are given in Table 1.

Platelet Studies

Autologous platelets were labeled with ^{111}In-oxine (Radiochemical Centre, Amersham, England), fully described elsewhere.[10,11] In brief, 200 to 300 mL of blood was collected in syringes containing acid-citrate dextrose NIH formula A (ACD). Platelet-rich plasma was prepared by centrifugation and was acidified with ACD to pH 6.2–6.5. The maximum number of platelets was harvested from the blood by washing platelets from the red cell layer four times. Platelets were labeled with ^{111}In-oxine in a saline medium, resuspended in autologous platelet-poor plasma, and reinjected only if they aggregated normally with ADP. Homologous platelets were labeled with ^{51}Cr-disodiumchromate as recommended by the International Committee for Standardization in Hematology.[6] ^{111}In-AP and ^{51}Cr-HP were injected simultaneously. Contamination of the platelet product with ^{111}In or ^{51}Cr-labeled plasma proteins and red cells was measured.[6]

In vivo distribution of ^{111}In-AP was quantitated with a scintillation camera and a computer-assisted imaging system as previously described.[8,11] Whole body and region of interest (ROI) ^{111}In-radioactivity was corrected for attenuation by the geometrical mean method, and ROI activity was expressed as a percentage of whole body activity. The initial (equilibrium) radioactivity was derived by back extrapolation of linear least-squares regression analysis of data, and the final organ radioactivity was determined at the patient's own platelet survival time.

Platelet survival was estimated from whole blood specimens collected at 5, 15, 30, 45, 60, 90, and 180 minutes after reinjection of

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Table 1. Patient Data and In Vivo Organ ^{111}In Radioactivity at Zero Time

Patients and Pattern of Platelet Sequestration	Sex/Age (yr)	Number of Pregnancies	Blood Platelet Count ($\times 10^{-9}$/L)	^{111}In Activity in Spleen (% of Whole Body)	^{111}In Activity in Liver (% of Whole Body)
Splenic					
1	F/16	0	18	32.1	25.9
2	F/34	1	95	29.8	12.5
3	F/68	9	88	22.1	12.9
4	F/16	0	119	46.9	12.3
5	F/25	0	60	33.5	23.9
Mean ± 1 SD			76 ± 39	32.8 ± 9.8	15.8 ± 6.7
Diffuse Res					
Normal recovery					
6	F/20	0	8	20.6	40.1
7	F/19	0	4	8.8	39.6
8	M/20	—	5	22.2	46.8
Low recovery					
9	M/20	—	5	22.4	47.8
10	F/16	0	7	32.9	38.5
Mean ± 1 SD	—	—	6 ± 2	21.4 ± 8.6	42.6 ± 4.4
Reference values	—	—	150–400	31.1 ± 6.1	9.6 ± 1.2

labeled platelets and thereafter as necessitated by the rate of disappearance of platelets from the circulation.[6] Blood volume was estimated,[12] and platelets were counted by phase microscopy, or, if more than 30 × 10^9/L, with an electronic particle counter. Mean platelet survival was calculated by a nonlinear, least-squares curve-fitting technique. A computer program fitted the data to the following mathematical models: weighted mean,[6] multiple hit,[13] Dornhorst,[14] Meuleman,[15] polynomial,[16] and α order.[17] All data points lying between equilibrium or maximum blood radioactivity, and 20% of this activity, were included. Curve fitting was visually inspected with a graphics display monitor. Goodness of fit of each of the mathematical models to the data was evaluated by calculating the SD of the data points around the regression curve. There was no significant difference in the goodness of fit with these models.[18] Therefore, only the results of the mean platelet survival time estimated with the multiple hit model are presented. In all patients, the clearance of platelets from the circulation was exponential, reflected by removal of platelets after a single "hit."

Platelet recovery in the circulation at equilibrium was derived by back extrapolation of the platelet survival curve to zero time. Platelet turnover was calculated from the blood platelet count and the platelet intravascular life span.[1] The correction factor for the splenic pool was 90% divided by the percentage of ^{111}In-AP quantitated to be present in the spleen at equilibrium.

Platelets from four normal subjects were labeled with ^{111}In and ^{51}Cr as described above. Platelet subpopulations in the platelet-rich plasma were separated by density on discontinuous arabinogalactan (stractan) gradients as described by Corash et al,[19] and the radioactivity was measured. Radioactivity in the four buoyant density subpopulations was expressed as a percentage of that of the total. Mean platelet volume was measured electronically with a multichannel analyzer.[19]

Elution of Radionuclide From Labeled Platelets

The in vivo release of ^{51}Cr and ^{111}In from platelets was measured by determining the radioactivity present in platelet-poor plasma of all the specimens collected for the estimation of mean platelet life span. This radioactivity, not associated with platelets or other cells, was expressed as a percentage of ^{111}In or ^{51}Cr whole blood radioactivity at equilibrium, normalized to 100%.

Statistics

The normal distribution of the data was assessed with the Shapiro-Wilk test. Paired or unpaired data were evaluated with either the t-test for different means or the nonparametric Wilcoxon's rank sum test where appropriate. The relationship between two variables was expressed as the correlation coefficient, r, and its significance was evaluated with the t test.

The percentage SD of the platelet turnover owing to the propagation of errors introduced by the measurements of the platelet count, the platelet life span, and the estimate of the splenic platelet pool size was computed by addition in quadrature of the fractional SDs.[20]

RESULTS

Pattern of ^{111}In-Labeled Platelet Destruction

The results of quantification of organ ^{111}In radioactivity at the end of platelet life span are given in Table 2.

The patients were separated into two groups on the basis of major organ or regional ^{111}In-AP radioactivity accumulation: five with a splenic sequestration pattern, and five with a diffuse pattern of platelet destruction in the RES. The distinction was based on the percentage of ^{111}In-AP in the spleen and the liver at the end of the platelet life span. The normal spleen-liver ratio is 1.4 ± 0.6. Those patients with a spleen-liver ratio ≥ 1.4 were classified as having "splenic sequestration," and those with a lower ratio were classified as having "diffuse RES sequestration." Two patients (9 and 10) of the latter group were segregated as a subgroup because they had exceptionally low recovery values and, as compared with others in this group, had relatively long platelet survival times (Table 3).

In the group with a splenic sequestration pattern, the mean spleen ^{111}In activity was 50.8% ± 13.6%, and that of the liver was 19.4% ± 7.1%. Splenic activity was significantly higher ($P < .025$), and that of the liver was significantly lower ($P < .025$), than that of controls (t test). In the group with diffuse RES sequestration, the mean hepatic ^{111}In activity of

Table 2. Sequestration of ^{111}In-Labeled Platelets at the End of Platelet Life Span

Patient and Sequestration Pattern	Organ or Region ^{111}In Radioactivity (Whole Body Activity) (%)			Ratio	
	Spleen	Liver	Remainder*	Spleen-Liver Ratio	Spleen-Remainder Ratio
Splenic					
1	33.6	24.4	41.6	1.4	0.8
2	61.9	26.6	11.3	2.3	5.5
3	41.6	12.1	43.6	3.4	1.0
4	66.3	11.4	22.2	5.8	3.0
5	50.5	22.6	27.2	2.2	1.9
Mean ± 1 SD	50.8 ± 13.6	19.4 ± 7.1	29.2 ± 13.6	3.0 ± 1.7	2.4 ± 1.9
Diffuse RES					
Normal recovery					
6	21.0	41.1	37.9	0.5	0.6
7	9.9	40.0	49.9	0.3	0.2
8	21.7	44.8	33.4	0.5	0.6
Low recovery					
9	22.5	47.6	29.7	0.5	0.8
10	35.5	39.1	29.3	0.9	1.2
Mean ± 1 SD	22.1 ± 9.1	42.5 ± 3.6	36.6 ± 8.5	0.5 ± 0.2	0.7 ± 0.4
Reference values (n = 6)	37.2 ± 8.8	29.2 ± 9.6	31.7 ± 8.3	1.4 ± 0.6	1.3 ± 0.5

*The remainder is ^{111}In radioactivity located neither in the spleen nor liver. This may be regarded as approximately reflecting bone marrow activity.

42.5% ± 3.6% was significantly higher ($P < .0005$), and the mean splenic activity of 22.1% ± 9.1% was significantly lower ($P < .025$), than that of normal subjects (t test).

The ^{111}In radioactivity other than in the liver or the spleen, designated "remainder" (Table 2), varied from patient to patient and did not reflect the pattern of platelet sequestration. Although the spleen-remainder ratio was lower in the diffuse RES sequestration group, this ratio was too variable to distinguish patterns of platelet destruction.

Distribution of the In Vivo ^{111}In Platelet Activity at Equilibrium

In the group with diffuse RES destruction, mean hepatic ^{111}In activity was four times higher ($P < .005$, t test) and that of the spleen was significantly lower ($P < .025$, t test) than normal (Table 1).

Platelet Recovery, Life Span and Platelet Turnover

Blood platelet counts, mean platelet survival, recovery in the circulation, and platelet turnover measurements as determined with ^{111}In-labeled and ^{51}Cr-labeled platelets are given in Table 3.

^{111}In-labeled platelets. Patients were reinjected with a mean of 11.8 MBq ± 5.3 (319 μCi ± 143) ^{111}In, labeling 20.7 × 10^8 ± 22.1 platelets. The blood platelet counts were much higher in the splenic sequestration group than in the diffuse RES sequestration group. A greater mean number of labeled platelets and more ^{111}In was thus reinjected per patient in the splenic sequestration group. Mean recovery of platelets in the circulation of all patients was 55% ± 25%, which was somewhat lower, but not significantly so ($P > .2$), than that of normal. In patients 6, 7, and 8, with diffuse sequestration of platelets, recovery was about normal. Patients 9 and 10 had very low recoveries.

Platelet size distribution in whole blood, platelet-rich plasma, and the labeled platelet-rich saline eventually injected was determined in three patients. Although there was a loss of approximately one-half of the platelets during the isolation and labeling procedures, mean platelet volume did not change significantly: whole blood 16 ± 2 fL, platelet-rich plasma 17 ± 2 fL, and platelet-rich saline 15.5 ± 2 fL.

The mean platelet life span, range 4.3 to 65.9 hours, of ^{111}In-AP was decreased in all patients and correlated with the platelet count ($r = 0.58$, $P < .05$). The mean platelet life span of the diffuse RES sequestration group was shorter than that of patients with splenic sequestration. The results, however, were variable, and patients 9 and 10 had platelet survival times that were much longer than those of the others in this group.

Mean platelet turnover of all patients was 53 × 10^9 platelets per liter per day ± 49 (range 4 to 114). The mean platelet turnover of the splenic sequestration group was significantly higher ($P < .005$, Wilcoxon test) than normal. Platelet turnover in patients with a diffuse RES sequestration pattern was variable and was markedly low in patients 9 and 10.

^{51}Cr-labeled platelets. Patients were reinjected with a mean of 2.8 MBq ± 0.8 (84 μCi ± 15) ^{51}Cr, labeling 135.2 × 10^8 ± 57.3 platelets. Mean platelet recovery in the circulation in all patients was 51% ± 21% and, although lower than normal, was not significant ($P > .5$). The mean recovery in the circulation of the diffuse RES sequestration group was much lower than normal in patients 9 and 10.

The mean life span of ^{51}Cr-HP was shortened to varying degrees (range 1.8 to 39.6 hours), and in the patients with diffuse RES sequestration it was shorter than in patients with splenic sequestration. Patients 9 and 10 were noteworthy exceptions. Mean platelet turnover of the whole patient group was normal, 93 × 10^9 platelets per liter per day ± 80 ($P > .1$). There was, however, a significant ($P < .005$, Wil-

Table 3. Isotope Data: Estimates of Platelet Recovery, Life Span, and Turnover

		^{111}In-Labeled Autologous Platelets					^{51}Cr-Labeled Homologous Platelets				
Patient and Sequestration Pattern	Blood Platelet Count ($\times 10^{-9}$/L)	Platelets Injected ($\times 10^{-9}$)	^{111}In Administered MBq(μCi)	Recovery in Circulation (%)	Life Span (h)	Turnover (10^{-9}pl/L/day)	Platelets Injected ($\times 10^{-9}$)	^{51}Cr-Administered MBq(μCi)	Recovery in Circulation (%)	Life Span (h)	Turnover (pl/L/day)
Splenic											
1	18	40.0	7.4(200)	51	5.0	115	180.8	3.2(120)	65	3.3	174
2	95	52.0	18.5(500)	65	65.9	44	132.0	4.1(150)	35	28.5	103
3	88	40.0	22.2(600)	76	20.8	117	122.8	3.0(112)	89	11.1	220
4	119	52.0	14.1(380)	58	37.6	129	130.1	4.1(150)	52	24.5	198
5	60	3.4	13.3(360)	79	35.6	55	67.1	2.4(90)	55	23.5	83
Mean ± 1 SD	76 ± 39	30.3 ± 24.8	15.1 ± 5.6 (408 ± 151)	66 ± 12	33.0 ± 22.6	92 ± 39	126.6 ± 40.4	3.4 ± 0.7 (124 ± 28)	59 ± 20	18.2 ± 10.6	156 ± 60
Diffuse RES											
Normal recovery											
6	8	3.5	9.3(250)	62	12.9	17*	83.7	2.7(100)	59	8.3	26
7	4	2.7	6.7(180)	88	4.3	22	106.1	2.7(100)	53	2.7	35
8	5	4.0	9.3(250)	40	7.2	19	82.0	2.2(80)	61	1.8	77
Low recovery											
9	5	4.5	5.6(150)	15	37.6	4	205.1	1.9(70)	16	24.6	6
10	7	5.2	11.1(300)	16	35.6	6	241.9	1.9(70)	28	39.6	6
Mean ± 1 SD		5.8 ± 1.6	8.4 ± 2.2	44 ± 31	19.5 ± 15.9	14 ± 8	144 ± 74.6	2.3 ± 0.4	43 ± 20	15.4 ± 16.3	30 ± 29
Reference values (±1 SD)	150–400	—	—	61 ± 12	226 ± 24	38 ± 6	—	—	68 ± 13	220 ± 24	35 ± 5

Abbreviation: pl, platelets.

*The percentage SD of platelet turnover introduced by the fractional SD of the platelet count (0.5), the platelet life span (0.1), and the estimate of the splenic pool size (0.06) was 26%.

coxon test) difference in the mean platelet turnover of the patients with different sequestration patterns.

Comparison of Mean Platelet Survival and Turnover Measurements With ^{111}In-AP and ^{51}Cr-HP

A mean of 8.0% ± 3.1% ^{51}Cr and 7.5% ± 6.5% ^{111}In was present in the plasma in which the labeled platelets were suspended. Contamination of labeled platelets with labeled red cells was low; 1.3% ± 1.1% and 1.1% ± 1.6% for ^{51}Cr and ^{111}In, respectively.

Mean survival of ^{111}In-AP was 26.3 hours ± 19.7, and that of ^{51}Cr-HP was 16.8 ± 13 hours. This difference was significant ($P < .005$, Wilcoxon test). This difference was evident whether women patients had had previous pregnancies or not. The difference influenced the estimate of mean platelet turnover: with ^{111}In-AP it was 53 × 10^9 platelets per liter per day ± 49, which was significantly ($P < .005$, Wilcoxon test) lower than the 93 × 10^9 platelets per liter per day ± 80 as estimated with ^{51}Cr-HP.

Labeling of Platelet Subpopulations

Labeling efficiencies with ^{111}In and ^{51}Cr of density-dependent platelet subpopulations separated on a stractan gradient, were similar ($P > .2$; *t*-test) (Table 4).

Plasma Radioactivity

The ^{111}In and ^{51}Cr radioactivity in platelet-poor plasma was measured for the duration of the platelet life span. The changes in radioactivity in plasma during the first nine hours after reinjection of labeled platelets are illustrated in Fig 1. ^{51}Cr activity was higher than that of ^{111}In ($P < .005$, *t* test) throughout the life span of the platelets, but the difference was more striking in the first few hours after reinfusion of labeled platelets.

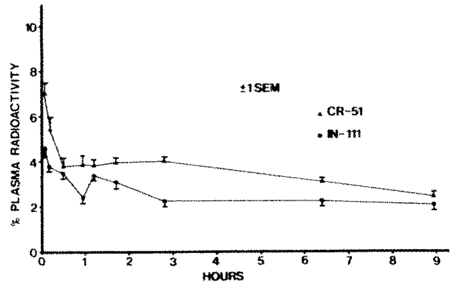

Fig 1. Plasma ^{111}In and ^{51}Cr radioactivity of blood samples taken to measure mean platelet survival time. ^{51}Cr plasma activity was significantly ($P < .005$) higher than that of ^{111}In. This is presumably due to in vivo elution of the label.

DISCUSSION

It has generally been stated that platelet turnover is increased in patients with ITP, and that this increase is especially evident when the blood platelet count is <50 × 10^9/L,[1-5] but not all authors agree.[9,21-24] A major difficulty thwarting the clarification of the problem is the inaccuracy of the measurement of platelet turnover in ITP. In ITP, it is difficult to measure three of the variables used in calculating platelet turnover: mean platelet life span, recovery of platelets in the circulation, and the blood platelet count. We have exploited the superior physical characteristics of ^{111}In and its high platelet labeling efficiency to minimize these difficulties. This radionuclide makes it possible to determine platelet survival and to image the in vivo distribution of autologous platelets quantitatively even in the presence of severe thrombocytopenia.[9,10] In addition, the size of the splenic pool may be determined accurately with quantitative imaging.[11,25]

It is convenient to consider our findings in the context of the in vivo distribution of the labeled platelets. In our previous study, we divided patients with ITP into three groups on the basis of the sites of platelet destruction: splenic, hepatic, and diffuse RES sequestration.[9] Because this grouping was somewhat arbitrary and there was considerable overlap, we have now grouped the hepatic and diffuse RES sequestration patients together. The division between the two major groups was based on the spleen-liver ratio and was clearcut. Although they had low spleen-liver ratios, patients 9 and 10 had low recovery values and relatively long mean platelet survival times; we have therefore separated them as a subgroup. Most of the platelets not destroyed in the liver or spleen, ie, the remainder, are sequestrated in the bone marrow.[8,9,25] Although the spleen-remainder ratio was lower in patients with diffuse RES platelet sequestration, this ratio neither identified another pattern of sequestration nor improved on the discriminatory value of the spleen-liver ratio. We observed a relationship between the blood platelet count and the sequestration pattern. Splenic sequestration was seen in patients with a relatively high platelet count, whereas diffuse RES sequestration occurred in those with low platelet counts. We therefore agree that the pattern of platelet sequestration is related to the severity of the disease.[4]

Table 4. Percentage of Distribution of Normal Platelets of Different Densities Labeled With ^{111}In and ^{51}Cr

	Stractan Density (g/mL) 1.084		Stractan Density (g/mL) 1.071		Stractan Density (g/mL) 1.066		Stractan Density (g/mL) 1.062	
Speciman	^{111}In	^{51}Cr	^{111}In	^{51}Cr	^{111}In	^{51}Cr	^{111}In	^{51}Cr
1	53.0	51.0	32.0	34.0	10.0	10.0	5.0	5.0
2	48.0	48.6	28.6	31.5	17.1	15.8	6.0	4.0
3	50.0	53.0	24.0	25.0	21.0	19.0	5.0	3.0
4	51.0	50.0	23.0	24.0	14.0	16.0	12.0	10.0
Mean ± 1 SD	50.5 ± 2.1	50.7 ± 1.9	26.9 ± 4.2	28.6 ± 4.9	15.5 ± 4.7	15.2 ± 3.8	7.0 ± 3.4	5.5 ± 3.1

In normal subjects, mean platelet survival estimated with ^{111}In and ^{51}Cr as cell labels is similar.[26] We now demonstrate that in ITP the survival of ^{111}In-AP is significantly longer than that determined simultaneously with ^{51}Cr-HP. This agrees with our previous view[8] and has recently also been found in moderately thrombocytopenic patients with ITP studied with ^{111}In- and ^{51}Cr-labeled autologous platelets.[27] It is unlikely that the discrepancy is due to isoimmunization because previous pregnancies or blood transfusions were not relevant in our patients. Neither is varying use of homologous *v* autologous platelets the explanation.[27]

It has been shown in vitro that small molecules such as ^{51}Cr are more readily lost from platelets than is ^{111}In-binding protein.[28] Our results, although not conclusive, suggest that this may occur in vivo. Plasma activity of ^{51}Cr was significantly higher than that of ^{111}In, especially during the early period after reinjection of labeled platelets. The in vitro contamination of labeled platelets with ^{51}Cr or ^{111}In in plasma was similar; therefore, the infusion of free radionuclide does not explain the difference in platelet survival times with the two isotopes. However, it is possible that the two radionuclides may be bound to different plasma proteins. This would affect the clearance rates of the isotopes from the circulation and is an alternative explanation for the results in Fig 1.

We also investigated the possibility that ^{51}Cr and ^{111}In do not label the circulating platelet population uniformly or to the same extent. Misleading platelet survival information could be obtained if one of the isotopes had a greater affinity for a specific platelet subpopulation. This is unlikely because ^{111}In and ^{51}Cr radioactivities were distributed equally in the platelet subpopulations separated on stractan. In addition, a select population of platelets with a different mean volume from that of whole blood platelets was not lost during harvesting and labeling.

Finally, platelets remaining in the circulation in ITP after a steady state has been reached may be relatively resistant to antibody injury. Autologous labeled platelets may thus have a longer mean survival time in the patient than does the heterogeneous platelet population of a normal donor. This has a precedent in autoimmune hemolytic anemia.[29] Our study has not excluded this possibility.

The estimate of the size of the splenic platelet pool will also influence the calculation of platelet turnover. The size of the splenic pool is considered normal in ITP,[26] but the estimates of the percentage of recovery of labeled platelets in the circulation vary from low[2] to near normal.[1] This discrepancy may be owing to the methods used in determining recovery. It is not clear whether it is more appropriate to estimate recovery by extrapolation of blood radioactivity values to zero time (as in ref. 1 and our study) or by measuring blood radioactivity at equilibrium.[2] Our data do not answer this question, but demonstrate that the estimate of recovery may be influenced by the pattern of platelet sequestration. If one assumes that the only significant exchangeable platelet pool is in the spleen, percentage of platelet recovery in the circulation and splenic radioactivity should total ~100%. In normal subjects, mean platelet recovery is 61% ± 12%, and at equilibrium 31.1% ± 6.1% of ^{111}In-labeled platelets are present in the spleen.[11] This was not so in ITP. In patients with a splenic sequestration pattern, mean recovery was 65.8% ± 11.8%, and the size of the splenic pool was estimated at 32.8% ± 9.8%, resembling that of normal subjects. In contrast, the group of patients with diffuse RES platelet sequestration had a mean ^{111}In-AP recovery of 44% ± 31% and a mean spleen pool of 21.4% ± 8.6%. This deficit may readily be accounted for by the very high liver ^{111}In-AP activity at equilibrium. It seems likely that in patients with diffuse RES sequestration of platelets many platelets are destroyed in the liver before attainment of equilibrium between the blood and splenic platelet pools.

All of the above factors should be taken into account when considering platelet turnover in ITP. We estimate that the propagation of errors introduced by the determination of the parameters used to calculate platelet turnover has a percentage SD of 26% in patients with a blood platelet count of 10×10^9/L. There was also marked variation from patient to patient as reflected by the large SDs. Therefore, it is clear that the physiological interpretation of results of kinetic studies in these patients should be guarded.

Notwithstanding our reservations, our results indicate that platelet turnover calculated with ^{111}In-labeled autologous platelets identifies a subset of patients with low platelet turnover, and that these patients have more severe disease as reflected by a low platelet count and diffuse RES sequestration of platelets. Platelet turnover measured with ^{111}In-AP was high in all patients with a splenic sequestration pattern. The mean increase was 2.2 times that of normal (range 1.2 to 3.0). In contrast, mean platelet turnover was decreased (mean 0.4 times normal) in all patients with diffuse RES sequestration of ^{111}In-AP, and especially so in patients 9 and 10. Mean platelet turnover estimated with ^{51}Cr-HP was significantly higher in both patient groups than platelet turnover measured with ^{111}In-AP. Our results are thus not in accordance with studies in which platelet turnover was found to be either normal or increased in ITP[1-4,30] but tend to agree with those of recent studies with ^{51}Cr-labeled platelets[21,23] and ^{111}In-labeled platelets.[9,24,27]

These results may be related to other findings difficult to reconcile with the concept that platelet production is always increased in ITP. There is some evidence that antiplatelet antibodies may damage megakaryocytes and impair megakaryocytopoiesis. Rolovic et al[31] have shown that megakaryocytopoiesis in rats with thrombocytopenia induced with heteroimmune antiplatelet serum is markedly altered. They demonstrate coexistent platelet destruction and defective thrombopoiesis in this experimental model of ITP. It has also been shown that the antiplatelet antibody of ITP binds to both platelets and megakaryocytes[32,33] and may impair platelet production at the level of the mature megakaryocyte.[23] It therefore seems plausible that the low platelet turnover demonstrated in our patients may be owing to antibody-mediated injury to the megakaryocytes. The more severe impairment of thrombopoiesis seen in patients with diffuse RES sequestration of platelets may be related to the finding that this destruction pattern typical of severe disease is owing to greater amounts of antibody bound to platelets.[34] It seems plausible that megakaryocytes may be damaged in such patients with high antibody levels.

It it evident that the application of the techniques of labeling autologous platelets with ^{111}In and the technology of quantification of the in vivo distribution of labeled platelets have provided new insights into the pathogenesis of ITP. However, they have also exposed new problems reflecting the complexities of the disease and the difficulties associated with the interpretation of the data.

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Purified Radiolabeled Antithrombin III Metabolism in Three Families With Hereditary AT III Deficiency: Application of a Three-Compartment Model

By E.A.R. Knot, E. de Jong, J.W. ten Cate, A.H.C. Iburg, Ch.P. Henny, T. Bruin, and J. Stibbe

Purified human radioiodinated antithrombin III (^{125}I-AT III) was used to study its metabolism in six members from three different families with a known hereditary AT III deficiency. Six healthy volunteers served as a control group. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and crossed immunoelectrophoresis (CIE) showed the purified AT III to be homogenous. Amino acid analysis of the protein revealed a composition identical to a highly purified internal standard. The specific activity was 5.6 U/mg. Analysis of plasma radioactivity data was performed, using a three-compartment model. Neither plasma disappearance half-times nor fractional catabolic rate constants differed significantly between patients and control subjects. The mean absolute catabolic rate in the patient group was significantly lower than that of the control group at 2.57 ± 0.44 and 4.46 ± 0.80 mg/kg/day, respectively. In addition, the mean patient α_1-phase, flux ratio ($k_{1,2}$ and $k_{2,1}$) of the second compartment α_2-phase and influx ($k_{3,1}$) of the third compartment were significantly reduced as compared with control values. It has been tentatively concluded that the observed reduction in the second compartment may be caused by a decrease in endothelial cell surface binding.

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ANTITHROMBIN III (AT III) has been shown to be a major inhibitor of activated serine proteases.[1,2] A high incidence of thromboembolic disease has been recognized in individuals with a hereditary deficiency of this inhibitory protein.[3,4] The causal mechanism of the hereditary AT III deficiency may be based on a singularly decreased synthetic capacity, increased consumption within the vascular compartment by ongoing insufficient inhibition of thrombin generation, differences in the distribution of the proteins over various compartments, or a combination of the three mechanisms.

To elucidate the influence of these hypotheses on the actual cause of this deficiency, metabolic studies with highly purified radiolabeled AT III are required. Metabolic studies thus far performed have provided insufficient information. In one study, plasma disappearance half-times of transfused human AT III concentrate were calculated.[5] The corrective effect of a possibly increased consumption of AT III by normalization of AT III plasma levels is a theoretical pitfall in this study. Other studies involving a total of four AT III-deficient patients used either small amounts of radiolabeled AT III concentrates with a low specific activity (3.2 U/mg protein),[6] a preparation with an unfavorable activity-antigen ratio of 0.5,[7] or an autologous preparation with unknown specific activity.[8] Preparations with a higher specific activity, 5.75 U/mg protein, were used by other researchers,[9] but in this case, studies were performed on normal subjects and patients with deep venous thrombosis. Theoretically, the maximum specific activity that can be obtained in purified AT III preparations is 7 U/mg protein. Specific activities influence the plasma disappearance half-times as reflected in the shorter half-life found in the study using the highly purified AT III preparation (67.2 ± 6.2 hours),[9] when compared with the studies using preparations with either unspecified or lower specific activities (81.6 to 115.2 hours).[6,7] Contamination with even trace amounts of proteins with a longer plasma disappearance half-time (eg, albumin) may cause considerable error.[10]

The present study therefore describes the AT III metabolism in six patients with a known hereditary AT III deficiency, using highly purified radiolabeled AT III (5.6 U/mg). Data analysis was based on a three-compartment model.

MATERIALS AND METHODS

Subjects. The study group was comprised of six patients with a hereditary AT III deficiency; patients were from three different families. The control group consisted of six healthy subjects (Table 1). Laboratory investigations in all individuals showed no proteinuria or abnormal liver functions. Of four members of the patient group, three had a medical history of thromboembolism demonstrated by V/Q scanning and/or impedance plethysmography. These patients received oral anticoagulant therapy at the time of the study. The biological and antigenic activity measured by chromogenic and immunological methods, respectively, showed reduced levels in all patients (Table 1). Crossed immunoelectrophoresis (CIE) of their plasma against the specific human AT III antiserum in the presence of heparin revealed normal AT III peak electrophoretic mobility.

The study was approved by the medical ethical and research committees, and informed consent was obtained from each individual before entry into the study. The investigation was carried out according to the declaration of Helsinki-Tokyo.

Laboratory procedures. Venous blood was collected in plastic tubes containing either solid dipotassium EDTA (1.5 mg/1 mL of blood) or trisodium citrate dihydrate (3.2 mg/1 mL of blood) using a 20-g Wasserman needle. Plasma was prepared by centrifugation at 1,700 g for ten minutes and was rendered platelet-poor by a second run at 12,000 g. Plasma samples were stored in small aliquots at −70 °C. Reference pooled plasma was obtained from 40 healthy volunteers (sex ratio 1:1). Biologic AT III activity was measured using a chromogenic substrate method.[11] Antigenic activity was measured using 1% agarose containing 20 μL of antiserum/1 mL.[12] Both rabbit anti-human AT III antiserum and total antiserum were purchased from Behringwerke A.G., Marburg Lahn, FRG. Standard curves were prepared using stepwise dilutions of citrated pooled plasma. Electrophoresis of these samples (5 μL) was performed at 10 V/cm per plate for 16 hours; subsequently, they were washed in saline and in distilled water, and were then dried and stained with Coomassie brilliant blue. CIE was performed according to Laurell,[13] as modified by Sas et al.[14] In the first dimension, 5 μL of plasma was

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Table 1. Clinical Data of Patients With Hereditary AT III Deficiency and Control Subjects

Patients	Sex	Age (Yr)	Weight (kg)	OAC*	History	AT III (U/mL) (%)	AT III (Ag/100 mL) (%)	AT III (Ratio)	AT III (mg/100 mL)	Plasma Volume (ml/kg)
D	F	25	57	+	DVT	47	70	0.67	9.80	40.75
S_1	M	27	65	+	DVT(?)	48	60	0.80	8.40	45.75
S_2	F	25	50	−	—	42	58	0.72	8.12	42.84
B_1	M	50	88	+	PE	43	64	0.67	8.96	42.02
B_2	F	25	55	−	—	58	67	0.86	9.38	41.29
B_3	M	34	77	+	DVT	53	64	0.82	8.96	43.52
Mean ± SD patients (n = 6)		31.0 ± 9.9	65.3 ± 14.5			48.5 ± 6.0	63.8 ± 4.1	0.75 ± 0.08	8.94 ± 0.62	42.69 ± 1.80
Mean ± SD controls		34.1 ± 6.7	76.2 ± 7.1			107.0 ± 9.0	116.5 ± 10.1	0.92 ± 0.07	15.64 ± 1.41	43.73 ± 1.02

DVT, deep venous thrombosis; PE, pulmonary embolism.

*Oral anticoagulant therapy.

electrophoresed at 15 V/cm with heparin (Thromboliquine, Organon, Oss, The Netherlands) in both buffer and agarose (16.6 U/mL). Electrophoresis in the second dimension took place for 12 to 14 hours at the same voltage with the agarose containing 20 μL AT III antiserum permillilter.

Purification of AT III. As a basic substance, human AT III concentrate (albumin free-DcA-107) obtained from KabiVitrum, Stockholm, Sweden, was used. This concentrate was previously used in other studies.[7] However, CIE of the concentrate showed several contaminants that potentially could interfere with the survival study. The concentrate was therefore further purified, using a heparin sepharose chromatography column (2.5 × 45 cm). The non-heparin binding fraction was removed by washing with phosphate-buffered saline (PBS) (pH 7.4). The heparin binding fraction was eluted with 1.5 mol/L of NaCl in PBS (pH 7.4). Subsequent sodium dodecyl sulfate-polyamide gel electrophoresis (SDS-PAGE)[15] using a Coomassie brilliant blue stain revealed one band with a mol wt of 67,000 daltons (Fig 1A). CIE of this purified AT III against whole human antiserum showed one precipitation arch (Fig 1B). The specific heparin cofactor activity was 5.6 U/mg. Amino acid analysis (D. Collen, Leuven, Belgium) showed a composition similar to that of a highly purified internal standard preparation (Table 2).

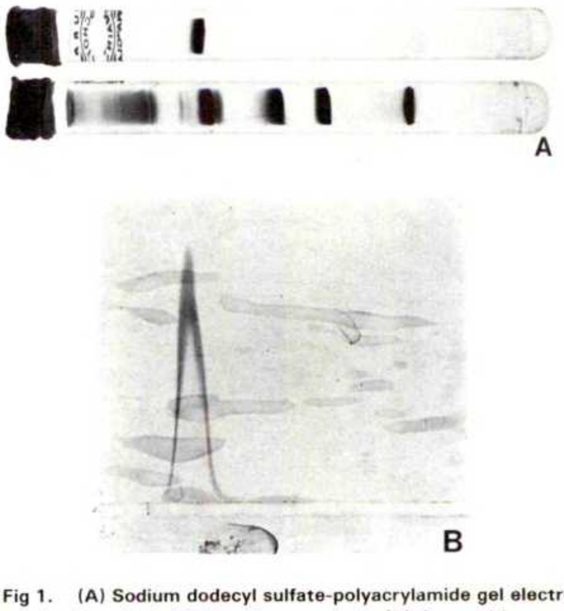

Fig 1. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified AT III and a standard. (B) Crossed immunoelectrophoresis of purified AT III in agarose containing whole human antiserum.

Preparation of radioactive tracer and in vitro characterization of ^{125}I-AT III. The purified AT III was radiolabeled with ^{125}Iodine using the Iodogen method.[16] ^{125}I-AT III was separated from the free iodine by gel filtration on Sephadex G 25. The ^{125}I-labeled tracer was subsequently applied to a heparin sepharose Column (1 × 8 cm). The non-heparin binding fraction was removed by washing by PBS (pH 7.4) and the heparin binding ^{125}I-AT III was eluted with 1.5 mol/L of NaCl in PBS (pH 7.4). The fractions were pooled, isotonized with sterile distilled water, treated with millipore filtration (0.45 μm), and stored in small aliquots at −70 °C. A specific radioactive activity of 1.3 to 4 μCi/1 mg AT III was obtained.

Each batch was tested and found to be negative for HbsAg (Ausria II) and endotoxins.[17] CIE[18] of a mixture of human plasma and ^{125}I-AT III against rabbit anti-human serum (Behringwerke), when stained for protein, showed a single precipitation arch. Its mobility was identical to that of the radioactivity peak depicted on autoradiography (Kodak XAR, Eastman Kodak Co, Rochester, NY).

Gel filtration (Sephadex G100) of a mixture of ^{125}I-AT III and normal human plasma showed a single radioactivity peak; elution patterns, as well as AT III antigenic and biological activity, were identical. The biological specific activity of the preparations was

Table 2. Amino Acid Composition of the Purified AT III and Internal Standard

	Batch I	Batch II	Internal Standard
Asx	11.68	11.30	11.98
Thr	5.60	5.65	5.73
Ser	9.04	8.68	8.33
Glx	13.47	13.28	13.02
Pro	4.63	4.72	5.47
Gly	5.06	5.05	4.69
Ala	8.33	9.10	8.07
Val	5.0	4.90	6.77
Met	2.14	2.30	3.12
Ile	4.22	4.39	5.47
Leu	9.75	9.76	10.15
Tyr	2.34	2.34	2.34
Phe	6.48	6.35	6.51
His	0.85	0.80	1.30
Lys	8.73	8.56	9.11
Arg	5.14	5.28	5.73

Tests were performed on a Beckmann 119 CL Analyzer; hydrolysis with 6MHCl and 40 hours in vacuum at 110 °C. Results are given as the percentage of each amino acid in the total bulk. The last column gives the composition of the AT III molecule as deducted from the complete amino acid sequence.

Table 3. Metabolic Data of Patients With Hereditary AT III Deficiency and Control Subjects

Patient	A_1	Asymptotic SD	α_1	Asymptotic SD	A_2	Asymptotic SD
D	0.324	0.138	0.255	0.0898	0.273	0.109
S_1	0.184	0.0094	0.856	0.126	0.658	0.0538
S_2	0.281	0.0710	0.289	0.0791	0.398	0.0566
B_1	0.325	0.196	0.602	0.0782	0.528	0.0658
B_2	0.304	0.0193	0.283	0.0180	0.413	0.0150
B_3	0.159	0.0101	0.635	0.0667	0.463	0.0210
Mean ± SD	0.263 ± 0.073		0.487 ± 0.247		0.456 ± 0.130	
Control subject						
P	0.143	0.0183	3.510	0.799	0.488	0.0194
R	0.069	0.0147	1.610	1.02	0.535	0.0156
S	0.112	0.0133	1.210	0.325	0.469	0.0192
H	0.116	0.0141	0.935	0.155	0.489	0.0225
E	0.238	0.0119	0.882	0.110	0.418	0.0410
J	0.115	0.0079	1.860	0.284	0.625	0.0239
Mean ± SD	0.132 ± 0.057		1.668 ± 0.979		0.504 ± 0.070	
	$P < .005$		$P < .005$		NS	
D	0.0591	0.0380	0.403	0.0941	0.0124	0.00218
S_1	0.0403	0.00358	0.158	0.0539	0.0089	0.00298
S_2	0.0518	0.0164	0.320	0.0806	0.0110	0.00221
B_1	0.0457	0.00859	0.148	0.0775	0.0096	0.00448
B_2	0.0532	0.00393	0.283	0.0178	0.0106	0.00560
B_3	0.0551	0.00405	0.378	0.0225	0.0105	0.00064
Mean ± SD	0.0509 ± 0.0068		0.282 ± 0.108		0.0105 ± 0.00120	
Control subject						
P	0.0660	0.0045	0.326	0.0218	0.0105	0.000641
R	0.0984	0.00915	0.396	0.0210	0.0120	0.000665
S	0.0748	0.00748	0.419	0.0246	0.0101	0.000645
H	0.0688	0.00722	0.345	0.0292	0.0102	0.000896
E	0.0590	0.00808	0.344	0.0476	0.0129	0.001370
J	0.0598	0.000330	0.260	0.0273	0.0128	0.000970
Mean ± SD	0.0711 ± 0.0146		0.348 ± 0.056		0.0114 ± 0.00130	
	$P < .005$		NS		NS	

unchanged after the labeling procedure. Turnover data obtained with this preparation could thus be assumed to reflect that of the total body AT III pool.

Turnover studies. Before injection and, subsequently, once daily throughout the course of the study, each subject received iodine capsules (100 mg) to avoid thyroidal uptake of ^{125}Iodine. Ten μCi ^{125}I-AT III was injected intravenously (IV) and blood samples were taken at 10, 30, and 60 minutes; at 2, 4, and 6 hours; and subsequently twice daily for 6 days. Urine was collected at 24-hour intervals. At the end of the study, the samples were counted for radioactivity using a Beckmann gamma counter (Beckmann Instruments, Inc, Fullerton, Calif).

Trichloroacetic acid precipitation was performed on both plasma and urine samples. Less than 2% of the radioactivity was recovered in the plasma supernatant, whereas no precipitable ^{125}I was found in the urine samples.

Tracer data. The initial values were obtained by curve-peeling on a home computer using a three-compartment model[19] and were then further analyzed on a VAX 11/750 computer by means of the program BMDP3R (V 2.1) (© 1979, the Regent of the University of California). Curve fitting as to the plasma radioactivity data was performed by a sum of three exponential terms:

$$(X(t) = A_1e^{-\alpha_1 t} + A_2e^{-\alpha_2 t} + Be^{-\beta t}.$$

The fractional catabolic rate constant ($k_{1,0}$), the transcapillary flux ratio's and plasma disappearance half times were calculated as follows:

$$k_{1,0} = 1/(A_1/\alpha_1 + A_2/\alpha_2 + B/\beta)$$

$$\begin{aligned} k_{2,1} = \tfrac{1}{2}|B \cdot (\alpha_1 - \beta) + A^2(\alpha_1 - \alpha_2) + \beta + \alpha_2 \\ + \sqrt{(\{B \cdot (\alpha_1 - \beta) + A_2(\alpha_1 - \alpha_2) + \beta - \alpha_2\}^2} \\ + 4A_2(\alpha_2 - \beta)(\alpha_1 - \alpha_2))| \end{aligned}$$

$$\begin{aligned} k_{3,1} = \tfrac{1}{2}|B \cdot (\alpha_1 - \beta) + A_2(\alpha_1 - \alpha_2) + \beta + \alpha_2 \\ - \sqrt{(\{B \cdot (\alpha_1 - \beta) + A_2(\alpha_1 - \alpha_2) + \beta - \alpha_2\}^2} \\ + 4A_2(\alpha_2 - \beta)(\alpha_1 - \alpha_2))| \end{aligned}$$

$$k_{1,2} = (k_{2,1} - \beta) \cdot (k_{2,1} - \alpha_2) \cdot (k_{2,1} - \alpha_1)/(k_{2,1} \cdot (k_{3,1} - k_{2,1}))$$

$$k_{1,3} = (k_{3,1} - \beta) \cdot (k_{3,1} - \alpha_2) \cdot (k_{3,1} - \alpha_1)/(k_{3,1} \cdot (k_{2,1} - k_{3,1}))$$

The plasma half-disappearance time = $\ln(2)/\beta$. The absolute catabolic rate constant (synthesis) is calculated from the fractional catabolic rate, the blood volume, and the AT III plasma antigen levels.

Statistical analysis. The Wilcoxon test for independent samples was used to establish the incidence of abnormal values outside the 95% confidence interval of the respective control values. A *P*-value of ≤.05 was considered significant. Results have been expressed in means ±1 SD.

RESULTS

The metabolic data of the patients and control subjects are depicted in Table 3. The following results were obtained in

Table 3. Metabolic Data of Patients With Hereditary AT III Deficiency and Control Subjects (Cont'd)

Patient	Transcapillary Flux $k_{1,2}$ (h^{-1})	Compartment 2 $k_{2,1}$ (h^{-1})	Transcapillary Flux $k_{1,3}$ (h^{-1})	Compartment 3 $k_{3,1}$ (h^{-1})
D	0.0573	0.184	0.0204	0.0390
S_1	0.1440	0.705	0.0125	0.0149
S_2	0.0593	0.218	0.0196	0.0286
B_1	0.0370	0.165	0.0420	0.0198
B_2	0.0605	0.209	0.0224	0.0272
B_3	0.0888	0.540	0.0193	0.0304
Mean ± SD	0.0745 ± 0.0379	0.337 ± 0.228	0.0227 ± 0.0100	0.0267 ± 0.0084
Control subject				
P	0.490	3.01	0.0256	0.0344
R	0.102	1.50	0.0401	0.0487
S	0.120	1.08	0.0290	0.0405
H	0.137	0.78	0.0290	0.0342
E	0.189	0.68	0.0206	0.0335
J	0.203	1.66	0.0194	0.0266
Mean ± SD	0.207 ± 0.144	1.45 ± 0.855	0.0273 ± 0.0075	0.0363 ± 0.0075
	$P < .01$	$P < .005$	NS	$P < .05$

Patient	Fractional Catabolic Rate of Plasma Pool per Day	Plasma Half-Life t½ (β) (h)	Absolute Catabolic Rate (mg/kg/day)
D	0.624	55.9	2.49
S_1	0.698	78.1	2.68
S_2	0.636	62.9	2.21
B_1	0.888	72.9	3.34
B_2	0.674	65.4	2.61
B_3	0.535	66.4	2.08
Mean ± SD	0.676 ± 0.118	66.9 ± 7.8	2.57 ± 0.44
Control subject			
P	0.566	65.8	4.18
R	0.624	57.6	5.11
S	0.499	68.9	3.37
H	0.583	68.0	3.80
E	0.703	53.9	4.93
J	0.780	54.0	5.38
Mean ± SD	0.626 ± 0.101	61.4 ± 7.0	4.46 ± 0.80
	NS	NS	$P < .005$

NS, not significant.

the control group: the plasma half-life was 61.4 ± 7.0 hours with a fractional catabolic rate of 0.626 ± 0.101 of the plasma pool per day. The plasma AT III concentration was 15.64 ± 1.41 mg/100 mL. The absolute catabolic rate was 4.46 ± 0.80 mg/kg/day. In the patient group, the following metabolic data were obtained: a plasma disappearance half-time of 66.9 ± 7.8 hours with a fractional catabolic rate of 0.676 ± 0.118 of the plasma pool per day; when compared with the control group, no intergroup differences were found. The plasma AT III concentration was 8.94 ± 0.62 mg/100 mL with an absolute catabolic rate of 2.57 ± 0.44 mg/kg/day, which was significantly reduced in comparison with control values ($P < .005$).

The α_1-phase was significantly lower when compared with the control values 0.487 ± 0.247 and 1.668 ± 0.979, respectively ($P < .005$).

The influx and efflux ($k_{2,1}$ and $k_{1,2}$) of the second compartment (0.337 ± 0.228 hour^{-1} and 0.0745 ± 0.0379 hour^{-1}, respectively) were significantly reduced when compared with the control values (1.45 ± 0.855 hour^{-1} and 0.207 ± 0.144 hour^{-1}, respectively) ($P < .005$ and $P < .01$). The α_2-phase and influx ($k_{3,1}$) of the third compartment (0.0509 ± 0.0068 and 0.0267 ± 0.0084 hour^{-1}, respectively) were also reduced as compared with the control values (0.0711 ± 0.0146 and 0.0363 ± 0.0075 hour^{-1}, respectively ($P < .005$ and $P < .05$).

DISCUSSION

Because it has been recognized that the hereditary AT III deficiency is associated with an increased risk of the development of thromboembolic complications, the interest in the AT III metabolism has expanded.[3,4] Following the introduction of purification by heparin affinity chromatography,[20] AT III concentrates have been made available for therapeutic use. Recently, these concentrates have been tested[21] and have been found to be impure, containing substantial amounts of altered AT III molecules in addition to other proteins.

CIE of the basic purified AT III used in another metabolic study[6] showed contaminations with many other trace amounts of proteins. Because of the presence of these contaminants, these metabolic studies using other coagulation proteins and/or antithrombin III should be critically reviewed.[10] Albumin contamination of AT III concentrates used in metabolic studies may influence the results of the plasma disappearance half-time of AT III due to the long disappearance half-time of the contaminant proteins (22.5 days).[22] Metabolic studies using such concentrates and/or roughly purified AT III are thus not a true reflection of the in vivo biological behavior of AT III. For the present study, we therefore further purified the AT III concentrate by repeated affinity chromatography until a protein with a specific activity of 5.6 U/mg was obtained. Its purity was further analyzed by CIE against total human antiserum and by amino acid composition analysis, showing a single precipitation arch and revealing a pure AT III preparation, respectively. For data analysis, a three-compartment model was used (previously described[23]), which was better than a two-compartment model. This was also found by other investigators who studied AT III metabolism in an animal experimental model.[24] The results of the plasma disappearance half-time in patients (66.9 ± 7.8 hour) did not significantly differ from the control group value. These half-lives, however, were shorter in those found in studies using radiolabeled AT III concentrates,[6,7] but comparable to the half-lives found by Collen et al.[9] The patient mean fractional catabolic rate was not significantly different from control values, indicating that there is no consumption in the central compartment. This included the two patients who did not receive oral anticoagulant therapy. When compared with the rates of the control group, the absolute catabolic rate (synthesis) was significantly reduced in all patients. In addition, a slower α_1-phase and a reduced flux ratio ($k_{1,2}$, $k_{2,1}$) of the second compartment were found in the patient group. The α_2-phase and influx ($k_{3,1}$) of the third compartment were also slower as compared with the control subjects.

Translation of the mathematical analysis of various compartments into the biological model revealed an association between the first compartment and the intravascular compartment. Taking into consideration that the equilibrium of the third compartment was reached after ~25 hours, this may be biologically identified as the extravascular compartment. If so, reduced $k_{3,1}$ shows a smaller efflux from the extra vascular compartment into the vascular compartment.

Translation of the second compartment into any physiological compartment may still be speculative, although some arguments are indicative of the possible binding of AT III to endothelial cells.[25] This is further substantiated by the fact that apparent plasma volumes calculated from isotope dilution measurement (dose divided by radioactivity of the blood samples after ten minutes) are substantially larger than the real plasma volumes, indicating that a proportion of the injection protein may have been rapidly transferred from the circulation to a non-plasma vascular compartment.[24] This non-plasma vascular compartment may be associated with the AT III fraction bound to the endothelial AT III cofactor that has been found on the endothelial surface.[24-26] The hypothetical association with circulating blood cells has previously been excluded.[24]

The hypothesis that the second compartment may be associated with endothelial binding sites can be supported by findings from our previously performed metabolic studies in patients with cirrhosis of the liver.[27] In these patients (without ascites), a significantly increased α_1-phase was found, and a higher apparent distribution volume correlated well with the severity of disease (enlargement of liver and spleen, demonstrated by scintigraphy). In patients with a hereditary AT III deficiency, the slower rate of reaching equilibrium of the second compartment may be explained by two hypotheses: (1) an increased second compartment, possibly based on an increased number of AT III cofactor binding sites; and (2) a decreased binding potential of AT III to these binding sites.

In conclusion, in patients with a hereditary AT III deficiency, the absolute catabolism takes place at a lower rate. There are no signs of AT III consumption within the intravascular compartment. The AT III plasma disappearance half-time was normal. A slower rate was found at which the equilibrium of the second compartment (endothelial binding) is reached as well as a slower influx to the vascular compartment from the extravascular compartment.

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Fetal to Adult Hemopoietic Cell Transplantation in Humans: Insights Into Hemoglobin Switching

By Th. Papayannopoulou, B. Nakamoto, F. Agostinelli, M. Manna, G. Lucarelli, and G. Stamatoyannopoulos

A 2-year-old boy with refractory acute leukemia (ALL) was transplanted with liver cells from twin fetuses of an 18-gestational-week age. Regeneration of hemopoietic cells was evident during the second week following transplantation when a cellular, predominantly erythroid, marrow was present. Studies of bone marrow and peripheral blood cells obtained 21 days posttransplant showed that bone marrow and peripheral blood BFU-E-derived erythroblasts displayed typical fetal patterns of globin chain synthesis ($\gamma/\gamma + \beta$ ratios: 0.87 to 0.98). In addition, all of the individually analyzed erythroid clones displayed a fetal type of globin program, suggesting that the presence of rare, partially switched clones was unlikely. Additional evidence supported the fetal phenotype of these progenitors. The iI expression of culture-derived erythroblasts was typical for fetal erythroid cells. As in fetal cells, fetal sheep serum influenced neither the globin nor the iI phenotypes, and the growth characteristics were as those observed in fetal liver cultures. That these fetal progenitors matured in vivo and produced cells with a fetal program was shown by the pattern of globin biosynthesis in bone marrow cells and peripheral blood reticulocytes ($\gamma/\gamma + \beta$ ratios: 0.85 to 0.95) at days 14 and 21 posttransplantation. These results indicate that the transplanted fetal cells, in spite of their proliferation and differentiation in the environment of the recipient, continued to express during the early posttransplantation period fetal patterns of globin, surface antigenic determinants, and growth and response to environmental modulation. The observations in this patient support the notion that hemoglobin switching is primarily controlled by a mechanism intrinsic to the stem cell.

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TRANSPLANTATION of hemopoietic cells into recipients of a different developmental stage can be used as a model system for studying the cellular control of hemoglobin switching.[1-6] Transplantation of fetal liver cells into adult recipients, carried out in sheep, has provided insights as to whether the stem cell or the environment is primarily responsible for the control of hemoglobin switching.[1,2,5,6] In humans, transplantation of fetal liver cells has been used for experimental treatment of children with aplastic anemia or leukemia.[7-9]

We had the opportunity to study the erythroid cells of a 2-year-old leukemic child who was transplanted with a suspension of cells from the liver of second trimester human twin fetuses. Globin expression was studied in the in vivo erythroblasts and reticulocytes of the recipient. In addition, in the present study, the globin programs, growth pattern, and expression of the iI antigenic system were assessed in erythroid colonies grown from the recipient's bone marrow and peripheral blood samples. Our analyses suggest that the recipient's cells expressed, both in culture and in vivo, a typical fetal program of globins. The iI determinants and growth characteristics were those expected for fetal progenitors. These results suggest that the donor cells continued to express a genuine fetal phenotype while proliferating and differentiating in the recipient's environment.

MATERIALS AND METHODS

The patient was a 2-year-old boy who, at the age of 10 months, was diagnosed as having acute lymphocytic leukemia (ALL). He was transplanted after second remission following standard preparative regimens.[7,8] The donor cells were from twin fetuses of an estimated 18-week gestational age. He received red cell and platelet support in the posttransplant period. A bone marrow sample, obtained 14 days following transplantation, provided morphological evidence of trilineage engraftment. The patient died at the 27th posttransplant day from sepsis.

Blood counts (HCT, red cells, WBC, platelets, reticulocytes) were determined at frequent intervals (Fig 1). Fetal hemoglobin and F-cell numbers were evaluated on day +21. Globin biosynthesis in peripheral reticulocytes or in bone marrow cells, after incubation with ^{3}H-leucine, was carried out on day +14 and +22. Bone marrow and peripheral blood samples obtained on posttransplant day 21 were sent to Seattle for the studies described below.

Buffy coat bone marrow cells and nonadherent mononuclear cells from peripheral blood were plated in plasma clot and methylcellulose cultures. The composition of the media was as described before,[10] with the exception that in some plates fetal sheep plasma or serum[11] was substituted for the fetal calf serum (FCS). Growth was monitored by inspection of the cultures at regular intervals.

For studying the cellular expression of globins in the in vitro-derived clones, plasma clot cultures at days 8 and 9 were double-labeled with anti-γ and anti-β chain monoclonal antibodies,[12] using previously described immunofluorescent staining procedures.[13] For measuring globin biosynthesis in clones maturing sequentially in culture, a large number of mature erythroid clones (over 100 per time point and culture condition) were lifted from the plates, pooled, and incubated with ^{3}H-leucine at any given time. Lysates from the incubated colonies were used for globin chain isoelectric focusing and measurements of globin chain biosynthetic ratios as described before.[14] For measuring globin biosynthesis in individual erythroid bursts, each burst was placed in a microfuge tube, the incubation mixture (containing leucine-free media and ^{3}H-leucine) was added, and the lysates from incubated cells were used for globin chain isoelectric focusing.

To study the expression of i and I antigens in culture-derived erythroblasts, colonies were lifted from the plates. After the cells were dispersed, they were reacted either with anti-i (anti-i Den) serum or anti-I (anti-I Ma) serum and counterlabeled with anti-human IgM-fluorescein isothiocyanate (FITC). Fluorescent intensity of the labeled cells was assessed with both microscopic examination of the preparations and with analysis of fluorescent profiles using a FACS (Ortho System 50H Cytofluorograf with a 2150 computer).

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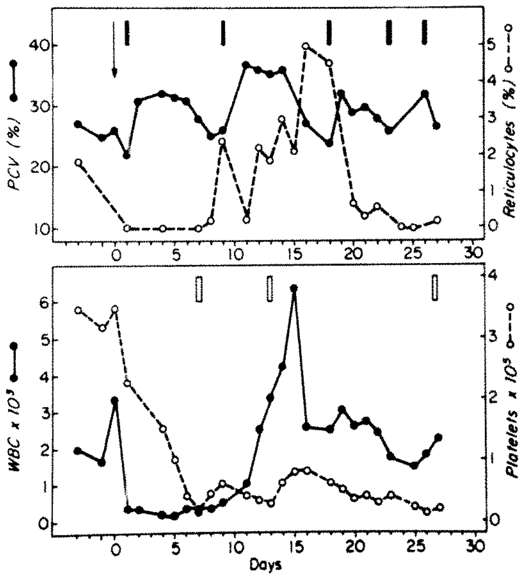

Fig 1. Hematological findings in the recipient. The arrow indicates day of transplantation. Solid bars, red cell transfusions; open bars, platelet transfusions. After the initial hemopoietic recovery with WBC and reticulocyte counts peaking at two weeks, the patient began to deteriorate clinically (fever, sepsis), and his peripheral blood counts started to decline.

Controls for the globin studies were obtained by culture of liver cells from human second trimester abortuses. As a control for posttransplantation globin expression, data from studies of bone marrow transplant recipients (11 aplastic anemia and 4 thalassemia major patients from 15 months to 33 years of age) 3 to 5 weeks posttransplant were used. Control cells for the iI studies were culture-derived erythroblasts from fetal liver BFU-E, from cord blood BFU-E, from blood BFU-E of a patient with homozygous β^+ thalassemia, and from two other patients with thalassemia major studied for 30 and 50 days after their bone marrow transplantation from HLA-identical siblings.

RESULTS

Globin biosynthesis in bone marrow (BM) and peripheral blood (PB) was done two and three weeks posttransplant. Both marrow samples were rich in erythroid precursors, although the marrow on day +21 was somewhat hypocellular. The $\gamma/\gamma + \beta$ ratios were 0.89 and 0.93 for BM and PB, respectively, at day +14; and 0.94 for BM at day +21 and 0.93 for PB at day +22. Circulating red cells from a posttransplant day 21 sample were stained with anti-γ chain monoclonal antibody. Twelve percent of the cells were brightly stained F cells. The discrepancy between number of circulating F cells and globin chain biosynthesis reflects the presence of transfused adult erythrocytes in the patient's blood.

Erythroid cultures were performed with BM and PB drawn on day -21. Bone marrow cloning efficiency at day 8 of culture was 605 colonies per 10^5 cells for the cultures containing FCS and 452 colonies per 10^5 plated cells in cultures containing fetal sheep serum. In contrast to the small-sized (CFU-E-like) colonies present in adult bone marrow cultures at these culture days, large burst-like colonies were present in the cultures of cells from the recipient. Such colony growth is characteristic of fetal liver cultures.[15] The cloning efficiency for BM BFU-E was $243/10^5$ for 11-day BFU-E and 75 and $160/10^5$ for 16-day BFU-E in plates containing FCS and fetal sheep serum, respectively. Circulating BFU-E were 28 and $85/10^5$ in FCS and fetal sheep serum cultures, respectively.

Globin biosynthesis in pooled BFU-E clones was measured in cohorts of erythroid bursts maturing sequentially in culture. All α/non-α chain ratios were balanced. In the bone marrow cultures $\gamma/\gamma + \beta$ ratios ranged from 0.9 to 0.98; in peripheral blood BFU-E cultures, they ranged from 0.87 to 0.96 (Figs 2 and 3). These ratios were in the range of fetal liver BFU-E cultures reported before[15-17] and of two fetal liver control cultures performed during the same time period. There was no increment in β globin biosynthesis when the erythroid bursts of the recipient were grown in fetal sheep plasma (Fig 3).

Globin expression in individual clones was evaluated with two approaches. First, plasma clot cultures were stained with anti-γ and anti-β chain antibodies, and the globin expression in individual bursts was assessed. There was weak labeling with the anti-β chain antibody, but a strong homogeneous labeling with anti-γ chain antibody (Fig 4). More than 100 labeled bursts were evaluated; all displayed the strong anti-γ chain labeling characteristic of the fetal erythroid bursts reported before.[16]

Second, globin chain biosynthesis was measured in individual erythroid clones (Figs 5 and 6). As shown in Fig 6, in all clones analyzed $\gamma/\gamma + \beta$ ratios were >0.85; analyses of single clones from cultures of two fetal liver controls provided similar results (Fig 6).

Figure 7 shows the FACS-derived profile of anti-i and anti-I labeling of erythroblasts from colonies of the recipient and of three controls. Characteristically, the patient's erythroblasts (Fig 7A) showed predominance of i expression over I expression; their FACS profile was typical of that obtained with fetal liver BFU-E-derived erythroblasts (Fig

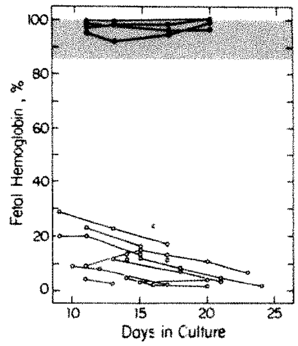

Fig 2. Globin biosynthesis in BFU-E cultures of the fetal liver recipient (●—●). The data represent values from sequentially maturing crops of bursts in peripheral blood and bone marrow (BM) cultures containing either fetal calf serum or fetal sheep serum or adult sheep serum. Dotted area shows the range of Hb F synthesis in cultures of fetal liver origin BFU-E. Data from 11 post bone marrow transplantation controls (AA patients) (○—○) were derived from single BM cultures done between 12 and 33 days posttransplantation.

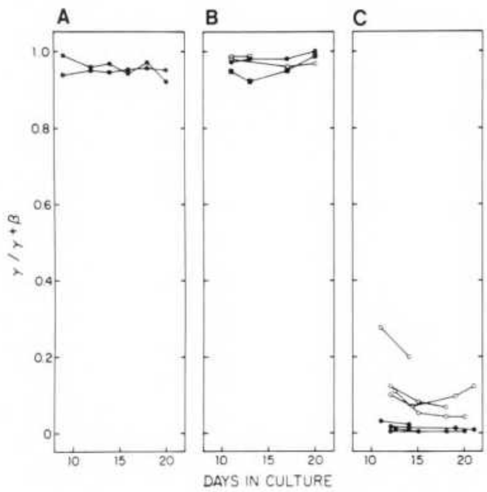

Fig 3. The effect of fetal sheep plasma (FSP) on $\gamma/\gamma+\beta$ globin ratios in cultures from fetal liver cells (A) (●—●); from the fetal liver (FL) recipient (B) (●—● bone marrow cultures, ■—■ peripheral blood cultures); and from posttransplantation controls (C) (●—●). Open symbols are for control cultures done with fetal calf serum in the media.

7B) and distinctively different from the profile of the cord blood BFU-E–derived erythroblasts (Fig 7C) or of erythroblasts from the BFU-E colonies of a patient with homozygous $\beta+$ thalassemia (Fig 7D) and of the two thalassemic patients in the early posttransplantation period (data not shown) which showed a typical adult pattern of iI labeling, similar to that shown in Fig 7D.

DISCUSSION

In this study, transplantation of fetal liver cells to a recipient with ablated erythropoiesis led to early engraftment and establishment of trilineage hemopoiesis, consistent with previous observations in patients transplanted with fetal liver cells.[7-9] Three weeks posttransplantation, detailed studies were done on the erythroid progenitors present in bone marrow and peripheral blood from the recipient as well as on erythroid cells maturing in vivo. The colonies produced in culture had features that were typical of fetal origin erythroid colonies. First, the $\gamma/\gamma+\beta$ biosynthetic ratio was in the range observed for fetal erythroid cells. Second, the pattern of globin chain synthesis was unchanged after the addition of fetal sheep serum, a finding that distinguishes the fetal erythroid colonies from the neonatal and the adult erythroid colonies.[11] Third, the growth characteristics of progenitors (colony number, size, and time of appearance in culture) were reminiscent of fetal liver progenitors. Fourth, the pattern of expression of iI antigenic system in culture-derived erythroblasts was typically fetal.[18] Taken together, these results reveal that a fetal program was encoded by the recipient's erythroid progenitors. That this program was also expressed in the cells maturing in vivo is suggested by the results of globin biosynthesis in peripheral blood reticulocytes and bone marrow cells, which revealed that the in vivo $\gamma/\gamma+\beta$ biosynthetic ratios were in the 0.89 to 0.94 range.

We interpret these observations to suggest that the fetal

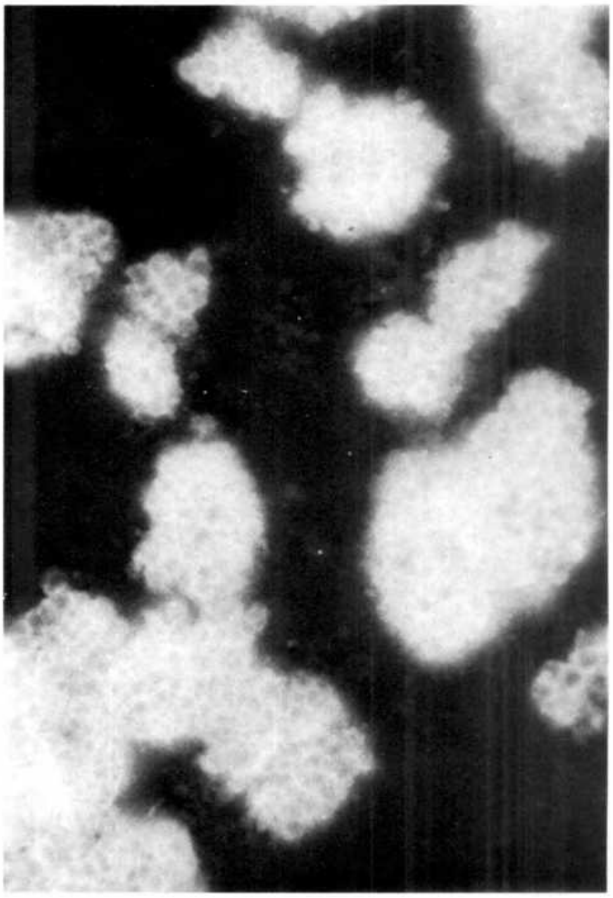

Fig 4. Fluorescent antibody labeling of eight-day erythroid clones from the cultures of the recipient's cells. The preparation was labeled with anti-γ chain monoclonal antibody. Strong homogeneous labeling with the anti-γ chain antibody was the characteristic finding from the analysis of the eight-day clones as well as of all bursts maturing at later culture days.

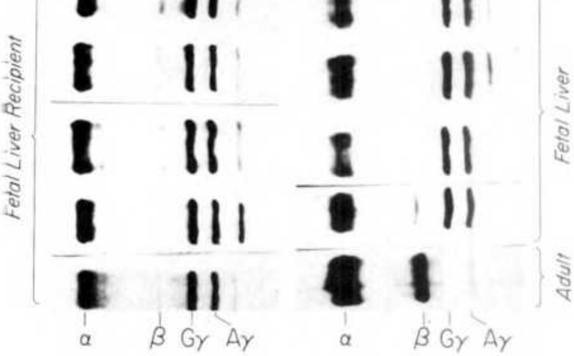

Fig 5. Globin chain isoelectric focusing of lysates from individual bursts from the recipient (A) and controls (B).

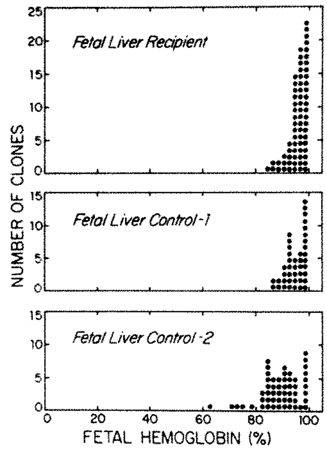

Fig 6. Hb F expression in individual BFU-E clones. Note that all the clones of the recipient show a globin program that is indistinguishable from that of fetal liver-origin control clones.

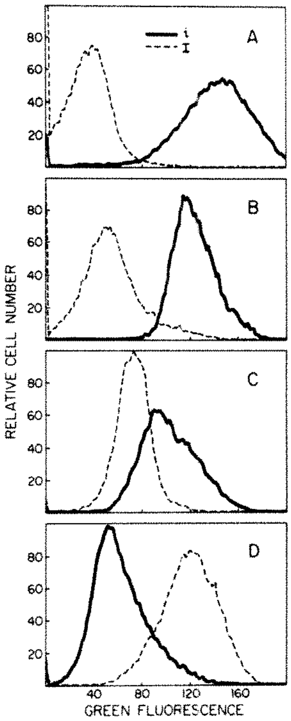

Fig 7. Profiles of anti-I (----) and anti-i (——) labeling. All of the cells are from BFU-E culture-derived erythroblasts. (A) Fetal liver transplant recipient; (B) fetal liver control; (C) neonate; (D) adult with homozygous $\beta +$ thalassemia.

cells that repopulated the recipient's hematopoiesis had failed to switch. It could be argued that our results do not reflect a failure of switching of the donor cells but rather a reactivation of fetal hemoglobin expression post bone marrow transplantation[19,20] or during the acute bone marrow expansion period.[20] Results of globin expression in BFU-E cultures of 15 recipients studied 12 to 33 days posttransplantation (Figs 2 and 3) showed that none of these cases had γ globin synthesis levels approaching the fetal range. In fact, in all cases β globin synthesis in BFU-E–derived erythroblasts exceeded that of γ globin. Furthermore, γ chain synthesis was readily inhibited when the posttransplant cells were grown in fetal sheep serum or plasma (Fig 3), and the pattern of comparative expression profiles of iI antigens in BFU-E–derived erythroblasts from bone marrow cell cultures of two of these recipients resembled that of adult cells. Hence, we think that it is unlikely that a posttransplantation reactivation of Hb F can explain the findings in the fetal liver recipient.

One of the possible mechanisms of control of γ to β switching is environmental induction.[1,3,16,21,22] It can be postulated that an environment, present during the fetal life, instructs the cells to express a fetal program of globin; alternatively, an environment established postnatally could be responsible for the expression by the erythroid cells of the adult globin program. It is also possible that a transient stimulus during the perinatal period could induce switching. The failure of the donor fetal cells to switch following the transplant suggests that the postnatal environment cannot induce switching in cells that have not been intrinsically programmed to switch. This interpretation could be challenged if one argues that switching was not observed in our patient because the environment in children of the recipient's age is not sufficient to induce the adult program in the donor cells. This argument implies that the transplants of young children will result in brisk production of fetal globin in the postengraftment period. Data from 15-month old to 4-year β-thalassemia homozygotes transplanted with bone marrow cells from young siblings have shown that in all instances Hb F levels in BFU-E-derived colonies are within the range observed for adult BFU-E (Fig 3). Furthermore, after transplantation of fetal liver cells in a 7-year-old recipient,[9] synthesis of Hb F in vivo was at levels similar to those we observed in the case reported here.

Although continuation of expression of a fetal program by the transplanted cells diminishes the likelihood that an adult environment alone can induce switching, it does not exclude the possibility that environmental induction plays a role in the control of switching. For instance, it may be argued that a fetal program is induced by the presence of a fetal environment whereas the expression of the adult program occurs in the absence of the fetal environment. Under this hypothesis, the failure of the donor cells to switch could be explained with the assumption that, together with the fetal hemopoietic cells, a cell population that created a "fetal environment" in the recipient's hematopoiesis was co-transplanted. The hypothesis of fetal environment has become less likely from the results of adult to fetus transplantation in the sheep,[2,4] which have shown that adult hemopoietic cells continue to express the adult globin program when they proliferate within a preswitch fetal environment. Additional evidence against this possibility is found in the results of replating of fetal liver origin early BFU-E progeny, which show continuous expression of fetal globin program in secondary plates in the absence of companion cells.[16]

The analyses of single erythroid clones either by immunofluorescence or by globin biosynthesis were done to test whether switching had occurred in a subpopulation of transplanted cells. Our findings suggest that if such a subpopulation existed in the recipient, it was very small in size (<1 in 200 assayable progenitors). The absence of single clones having an adult program is of relevance to the clonal evolution models of switching. Such models have been first proposed to explain switching from embryonic to definitive globin formation in birds and amphibia[24] and subsequently expanded to the explanation of switching in humans.[25–27] In the case of γ to β switch, the simplest assumption is that a fetal stem cell lineage is replaced by an adult stem cell lineage as development proceeds. Lineage models require a number of additional assumptions. For instance: the proliferative behavior of lineages could be controlled intrinsically by programs linking extinction or expansion of a lineage to developmental time, or expansion or "dormancy" of a lineage could be controlled by the prevailing environment. Our results are relevant only to a lineage model that also assumes a selective advantage, of each of the two putative stem cell lines, in either a fetal or in an adult environment. With such a model, the putative "adult" stem cells coexist with the fetal stem cells during the early stages of development, but remain dormant. If that were the case, expansion and differentiation of the adult stem cell lineage and extinction of the fetal lineage would be expected once the cells were placed in a postnatal environment. The absence of adult clones in our recipient argues against this hypothesis. Our results cannot exclude the possibility that an adult lineage exists but had migrated out of the liver of the 18-week fetuses, or that this lineage was present in the transplanted cells but could not proliferate because the developmental signal for its proliferation was not turned on. No permanent grafts have been obtained following transplantation with fetal liver cells; patients either die early or they survive longer with autologous marrow reconstitution (G. Lucarelli, unpublished observations). Thus, one could argue that the early trilineage engraftment in these patients is derived from preprogrammed committed progenitors rather than pluripotent progenitors, the latter being the only ones susceptible to reprogramming by the new environment. This discussion points to some of the inherent difficulties in interpreting the results of the existing in vivo experiments, in view of the multiple hypotheses that can be proposed for the control of switching.

Fetal to adult transplantations in sheep have shown that the developmental time from which the donor fetal cells derive is a variable that determines whether the engrafted cells will or will not switch.[5,6] Thus, cells from early fetuses fail to switch, whereas cells from later fetuses do switch; the rate of switching appears to depend on the developmental age of the fetus.[5,6] Such results support a purely intrinsic mechanism of switching, perhaps controlled by an intrinsic developmental clock,[5,16] or a model assuming that developmental time changes the ability of the cells to interact with environmental stimuli.[11] Our results are compatible with either of the latter two models, since it can be assumed that the donor fetal cells did not switch because their signal of switching or their ability to interact with their environment had not yet been activated.

ACKNOWLEDGMENTS

We thank Drs Rainer and Beverly Storb, as well as Mrs Cheryl Boyd for providing us with the samples of aplastic anemia patients and Dr E. D. Thomas for the samples of thalassemic patients.

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Progressive Dysfunction of Monocytes Associated With Iron Overload and Age in Patients With Thalassemia Major

By Isidro J. Ballart, María E. Estevez, Luisa Sen, Roberto A. Diez, Julia Giuntoli, Susana A. de Miani, and Jorge Peñalver

We evaluated phagocytic and lytic activities of peripheral blood monocytes (PBMo) from patients with thalassemia major(ThP) using *C pseudotropicalis* as the target. PBMo from ThP showed decreased lytic activity ($P < .001$), whereas the phagocytic activity did not differ from that of the controls. Significant inverse correlations were found between lytic activity of PBMo and age of patients ($r^2 = .47$; $P < .01$) and also between lytic activity and serum ferritin levels ($r^2 = .65$; $P < .001$). No association was found between lytic activity and other variables (blood transfusion regimens, therapy with desferrioxamine, liver damage, and the presence of sHBAg). Splenectomy showed no positive effect on PBMo functions from ThP. Our results suggest that PBMo from ThP have an intracellular defect in their microbicidal mechanisms associated with iron overload. This cell dysfunction could be responsible, at least in part, for the increased susceptibility to infections reported in ThP.

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PATIENTS WITH homozygous β thalassemia require regular blood transfusions to prevent severe, symptomatic anemia.[1] With time, however, the benefits of this regimen are gradually overshadowed by the effects of the iron overload that is inevitably induced.[2] In these patients iron accumulation in many organs, including the heart, liver, spleen, and endocrine glands, indicates a poor prognosis.[3] Splenectomy performed for the hypersplenism which often occurs in this disease renders these patients more susceptible to infections.[4] Many reports show alterations in both humoral and cellular immunity in this disease, although some of them are discordant.[5-14] Macrophages play a major role in both host defense and iron storage.[15] Although impaired functions of the mononuclear phagocytes should be expected in this disease, very little is known about them.[16,17] In an initial work, we demonstrated that the lytic activity of polymorphonuclear (PMN) leukocytes from thalassemic patients was markedly decreased, whereas their phagocytic function was normal.[5] The purpose of this investigation was to study the phagocytic and microbicidal activities of peripheral blood monocytes (PBMo) from patients with thalassemia major.

We also evaluated the effect of splenectomy on these functions and attempted to determine whether there was any association between phagocytosis and intracellular killing by monocytes and several clinical features of the thalassemic patients, including age, serum ferritin levels, liver damage, and treatment with chelating agents.

MATERIALS AND METHODS

Patients and controls. We studied 22 patients with homozygous β thalassemia, 10 females and 12 males, whose ages ranged from 1 to 24 years. Sixteen of the patients were nonsplenectomized (7 females and 9 males with ages ranging from 1 to 23 years) and 6 were splenectomized (3 females and 3 males with ages ranging from 12 to 24 years). Six of the 16 nonsplenectomized patients underwent splenectomy during the course of this study and were also evaluated after the removal of their spleens. Eight patients were under treatment with iron chelators (deferoxamine, 25 to 45 mg/kg, administered by continuous subcutaneous infusion using a portable pump). Eleven patients were under a "hypertransfusion" program to maintain their hemoglobin levels at >10 g/dL; the other 11 patients were under a "supertransfusion" program, to maintain their hemoglobin levels at >11.5 to 12 g/dL. The diagnosis of thalassemia major was made at the Hospital de Niños de Buenos Aires by standard hematological methods, hemoglobin analysis, and family studies. Adult patients and parents of the children with thalassemia gave their consent prior to entering the study. Blood samples were obtained immediately prior to transfusion of the patients. Our control group consisted of 19 normal subjects (9 females and 10 males with ages ranging from 3 to 46 years). Among them were nine normal children who were studied while undergoing routine blood analysis prior to plastic surgery; parental consent was obtained.

Preparation of yeast cells. We used *C pseudotropicalis*, strain 565, obtained from the Laboratory of Parasitology, Medical College of Paris. The yeast cells were cultured for eight hours at 37 °C on Saboureaud glucose-agar slants and were then collected, washed in Hanks' balanced salt solution (HBSS), and counted. To opsonize the yeast cells, 100 μL of pooled noninactivated normal human AB serum was added per 5×10^6 yeast cells; after several minutes, TC 199 medium (GIBCO, Grand Island, NY) was added to a final concentration of 5×10^6 yeast cells per milliliter.

Isolation of mononuclear cells and study of monocyte functions. Mononuclear cells were isolated from defibrinated peripheral blood by Ficoll-Hypaque density gradients (d = 1.077).[18] Cells at the interphase were collected, washed twice with HBSS, and resuspended in RPMI 1640 medium (GIBCO). Smears were stained with May-Grünwald-Giemsa. The percentage of monocytes after Ficoll-Hypaque separation ranged between 5% and 23%. In a previous study, we evaluated the optimal conditions for adherence of human PBMo.[19] We found that the maximum number of cells adhered to the coverslips when 2.5 to 3×10^6 cells per milliliter in RPMI 1640 with 20% of pooled heat-inactivated normal human AB serum were cultured in Leighton tubes for 18 to 24 hours. Ninety percent to 95% of the adherent cells were monocytes.

After separation with Ficoll-Hypaque, the cells were cultured as above, using the same lot of pooled heat-inactivated normal human AB serum for patients and for normal controls. At the end of the incubation period, supernatants were removed, and glass coverslips were washed twice with warm TC 199 medium. One milliliter of the opsonized yeast cells was added to each Leighton tube. The tubes were incubated at 37 °C and phagocytosis and lysis were allowed to

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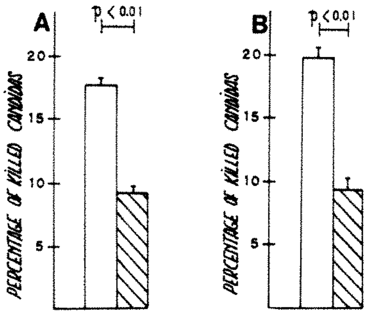

Fig 1. **Monocyte lytic activity from nonsplenectomized thalassemic patients (n = 16) and normal controls (n = 19) after (A) 30 and (B) 60 minutes of incubation with *C pseudotropicalis*. Bars represent $\bar{x}$ ± SEM. □, normal controls; ▨, thalassemic patients.**

proceed for 30 and 60 minutes. The extracellular yeast cells remaining in suspension were removed by gently washing the coverslips three times with warm TC 199 medium. To evaluate *Candida* phagocytosis and killing by adherent cells, coverslip monolayers were examined morphologically with Giemsa staining. Phagocytosis was expressed as the number of *Candida* cells ingested per 100 monocytes, and lytic activity was expressed as the percentage of ghost images (dead yeast cells) per 100 ingested *Candida* cells. The evaluation was done under blind conditions.

Serum ferritin concentration. Serum ferritin levels were determined by a two-site immunoradiometric assay based upon the method of Addison et al[20] as modified by Miles et al,[21] using a commercial kit (RAMCO, Houston, Tex).

Statistics. The student *t* test for independent groups and nonlinear correlation test were performed.

RESULTS

Phagocytic and lytic activities of monocytes from nonsplenectomized patients with thalassemia major. We studied the ability of PBMo from thalassemic patients to kill *C pseudotropicalis* after 30 and 60 minutes of incubation with the yeast. The percentages of intracellular killing were ($\bar{x}$ ± SEM) 8.08% ± 0.56% after 30 minutes and 9.5% ± 0.6% after 60 minutes as compared with 18.2% ± 0.3% and 20.0% ± 1.5% in normal controls (Fig 1A and B). The lytic activity of the monocytes from thalassemic patients was statistically decreased as compared with normal controls at both incubation periods ($P < .001$).

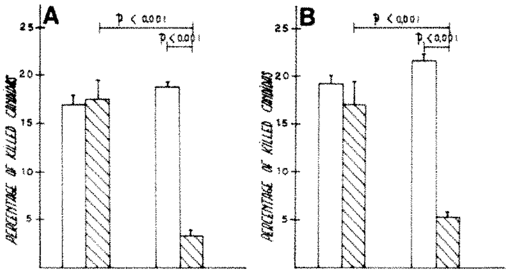

Fig 2. **Comparison of the lytic activities between thalassemic patients and normal controls <10 years or ≥10 years of age after (A) 30 and (B) 60 minutes of incubation with *C pseudotropicalis*. Bars represent $\bar{x}$ ± SEM. □, normal controls; ▨, thalassemic patients.**

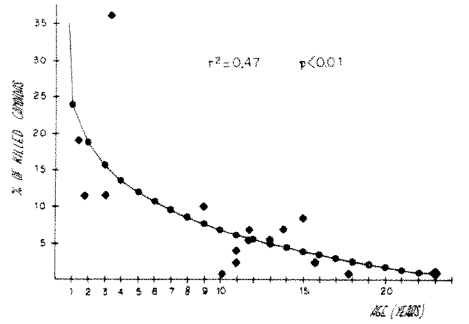

Fig 3. **Correlation between monocyte lytic activity and ages of 16 thalassemic patients studied before splenectomy.**

In order to assess whether there was any relationship between the impairment in the lytic function and the ages of patients, we divided the patients and controls into two age groups: <10 years old and ≥10 years old. As shown in Fig 2A and B, the percentages of killed *candida* cells were ($\bar{x}$ ± SEM): thalassemic patients aged <10 years: 17.7% ± 4.8% and 17.3% ± 5.4% at 30 and 60 minutes, respectively; patients aged ≥10 years: 3.7% ± 0.8% and 5.6% ± 1.2%; normal controls aged <10 years: 17.3% ± 2.3% and 19.3% ± 2.9%; and controls aged ≥10 years: 19.0% ± 1.6% and 21.4% ± 1.8% at 30 and 60 minutes, respectively. According to these results, the ability to kill yeast cells in thalassemic patients aged <10 years was similar to that observed in the age-matched control group, whereas in patients ≥10 years, the lytic activity was significantly decreased as compared with that of age-matched controls ($P < .001$). The statistical analysis of the association between the lytic function of the monocytes and the age of the patients showed an inverse correlation between both variables ($P < .01$) (Fig 3).

To assess whether the age-related defect in the lytic activity of the monocytes was associated with iron overload, we determined the serum ferritin concentration in 13 patients. As shown in Fig 4, an inverse correlation between lysis and serum ferritin levels was found ($P < .001$).

We also determined whether any association existed between monocyte lytic activity and therapy with chelating agents. No differences were found between thalassemic patients who were under treatment with deferoxamine for at least one year and those who were untreated (data not

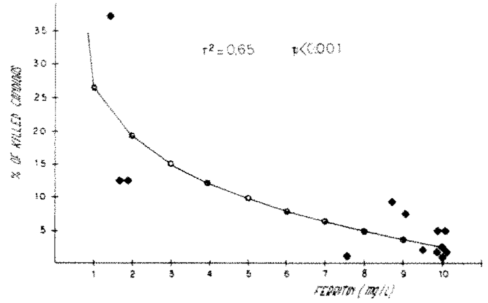

Fig 4. **Correlation between monocyte lytic activity and serum ferritin levels of 13 thalassemic patients studied before splenectomy.**

Table 1. Phagocytosis of *C pseudotropicalis* by Monocytes From Nonsplenectomized Thalassemic Patients and Normal Controls

Subjects Studied	Phagocytosis* (30 Min)	Phagocytosis* (60 Min)
Thalassemic patients		
Total (N = 16)	411 ± 19	460 ± 21
Age <10 yr (n = 5)	385 ± 31	418 ± 41
Age ≥10 yr (n = 11)	423 ± 24	480 ± 24
Normal controls		
Total (N = 19)	472 ± 25	492 ± 24
Age <10 yr (n = 9)	433 ± 37	463 ± 35
Age ≥10 yr (n = 10)	507 ± 32	518 ± 27

*Number of ingested *Candida* cells per 100 adherent cells ($\bar{x}$ ± SEM).

shown). Neither were any differences found between patients receiving supertransfusions and those receiving hypertransfusions; no association was found with other clinical parameters, including liver damage (as measured by liver size and serum glutamic pyruvate transaminase), serum proteins, or the presence or absence of surface hepatitis B antigen (sHBAg) (data not shown).

Table 1 shows phagocytosis by PBMo from thalassemic patients as compared with those of normal controls. No statistically significant differences were found between them after 30 or 60 minutes. Neither were differences found between patients younger or older than 10 years of age.

Effects of splenectomy on the monocyte functions. To evaluate the effect of splenectomy on the phagocytic and lytic activities of the monocytes from patients with thalassemia, we studied 13 splenectomized thalassemic subjects, six of whom had been previously studied before undergoing splenectomy. No differences were found in phagocytic or lytic functions between the splenectomized and nonsplenectomized patients (Table 2). The six patients studied before and after splenectomy showed no differences in phagocytosis; the already impaired lytic activity decreased even more after the removal of the spleen, being statistically significant at 30 and 60 minutes ($P < .05$) (Table 3).

DISCUSSION

Our study demonstrates that PBMo from nonsplenectomized patients with homozygous β thalassemia have an age-related defect in their microbicidal activity against *C pseudotropicalis* with a preserved phagocytic function. The deficiency in the intracellular killing of *Candida* cells is associated with iron overload and is not corrected by splenectomy.

There is evidence that patients with Cooley's anemia are particularly prone to infections, particularly after splenectomy.[4,22-24] In these patients, *S pneumoniae* is a major pathogen. Elimination of *S pneumoniae* from the bloodstream depends upon a correct humoral response and the integrity of the reticuloendothelial system.[25] Several authors have reported abnormalities in both humoral and cellular immunity in thalassemia major, but some of these results are discordant.[5-14] The few reports on the mononuclear phagocyte system in thalassemia evaluated the reticuloendothelial phagocytic capacity by the clearance of radiolabeled materials[16,17] and found a borderline low value. To assess the monocyte functions, we selected *C pseudotropicalis* as a target microorganism because these yeast cells are killed mainly through oxygen-dependent, myeloperoxidase-independent lytic mechanisms,[26] which are the main microbicidal systems that operate in mononuclear phagocytes.[27]

The alteration found in the lytic system of PBMo from thalassemic patients is coincident with that previously reported in PMN cells.[5] The candidacidal activity against *C pseudotropicalis* directly correlates with the chemiluminescence response evoked by stimulation with opsonized zymosan in PMN and monocytes (I. J. Ballart et al, manuscript in preparation). Preliminary data in monocytes from two thalassemic patients showed that both had a decreased candidacidal activity (<2%) and a very low monocyte chemiluminescence in response to opsonized zymosan stimulation. The defect found in the myeloperoxidase-independent microbicidal mechanisms could be caused by iron overload. It is known that patients with thalassemia major develop iron overload as a consequence of regular blood transfusions and ineffective erythropoiesis.[2] Iron storage increases with the age of the patients. Serum ferritin levels provide a useful estimation of iron storage.[28] It has been shown that iron interferes with bactericidal activity (by reducing H_2O_2) and phagocytosis (by the spontaneous generation of free radicals which damage the cells).[29,30] We found that serum ferritin levels were inversely correlated with the microbicidal activity of the monocytes and that the lytic activity decreased as the patients grew older. These findings suggest an association between iron overload and the impairment in the lytic activity of monocytes. Iron overload may also affect phagocytosis, ie, in idiopathic hemochromatosis.[31] Although the phagocytic activity of PBMo from patients with thalassemia tended to be lower than that of normal controls, no statistical significance was found. However, we cannot dismiss the possibility that the apparent differences found in killing reflect, at least in part, differences in complete ingestion.

Table 2. Comparison of Phagocytic and Lytic Activities From Nonsplenectomized and Splenectomized Thalassemic Patients ≥ 10 Years of Age

Patients Studied	Phagocytosis* (30 min)	Phagocytosis* (60 min)	Lytic Activity† (30 min)	Lytic Activity† (60 min)
Nonsplenectomized (n = 11)	423 ± 24	480 ± 24	3.7 ± 0.8	5.6 ± 1.2
Splenectomized (n = 12)	417 ± 15	441 ± 25	2.3 ± 0.6	1.7 ± 0.5

*Number of ingested *Candida* cells per 100 adherent cells ($\bar{x}$ ± SEM).

†Percentage of intracellularly killed *Candida* cells ($\bar{x}$ ± SEM).

Table 3. Phagocytosis and Lytic Activity of the Monocytes From Six Thalassemic Patients Studied Before and After Splenectomy

	Phagocytosis*		Lytic Activity†	
	(30 min)	(60 min)	(30 min)	(60 min)
Before splenectomy	417 ± 24	441 ± 30	3.1 ± 1.0	4.4 ± 1.7
After splenectomy	403 ± 29	418 ± 47	0.7 ± 0.2	1 ± 0.3

*Number of ingested *Candida* cells per 100 adherent cells ($\bar{x}$ ± SEM).

†Percentage of killed *Candida* cells within the adherent cells ($\bar{x}$ ± SEM).

The treatment with chelating agents in patients with thalassemia did not modify the lytic function or serum ferritin levels; neither was any association found between cell dysfunction and blood transfusion programs, liver damage, serum protein concentration, and the presence of sHBAg. Splenectomy did not improve the monocyte function. On the contrary, the already impaired lytic activity became worse after removal of the spleen.

In conclusion, monocytes from patients with thalassemia major have an intracellular defect in their microbicidal mechanisms, associated with iron overload. Neither splenectomy nor chelating agent therapy could revert this impairment. This cell dysfunction could be responsible, at least in part, for the increased susceptibility to infections reported in thalassemia major.

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Influence of Oxygen Tension on the Viscoelastic Behavior of Red Blood Cells in Sickle Cell Disease

By Gerard B. Nash, Cage S. Johnson, and Herbert J. Meiselman

Although the rheological behavior of sickle cell suspensions and of hemoglobin S solutions is known to be strongly dependent on oxygen tension (Po_2), little data exist concerning the influence of Po_2 on the viscoelasticity of 2ndividual HbSS RBC. We have used micropipette aspiration techniques to test the deformation response of both HbSS and control HbAA RBC over a wide range of Po_2 at 23 °C. Sickled, spiculed HbSS cells were present for $Po_2 \leq$ 35 mm Hg; for a number of these cells, the deformation response was essentially elastic and an effective membrane rigidity (EMR) was calculated. EMR increased with decreasing Po_2 and was ~5 to 50 times higher than the equivalent rigidity of oxygenated HbSS RBC. In addition, the rate of membrane deformation was very slow for sickled cells; the half-time for the deformation process increased as Po_2 was lowered and was about two orders of magnitude longer than the equivalent time for normal RBC. Other sickled cells exhibited plastic deformation when subjected to comparable deforming forces and experienced irreversible membrane deformation and budding. At all Po_2 levels tested, some HbSS RBC remained as discocytes; these cells had normal membrane elasticity and membrane viscosity. Furthermore, changes in Po_2 did not affect the membrane properties of HbAA RBC. Thus, gross abnormalities in the deformation response of HbSS RBC were only detected after morphological sickling had occurred. These abnormalities most likely arose from changes in the cytoplasmic HbS viscoelasticity and, if present in vivo, would be expected to impair the flow of HbSS cells in the microcirculation.

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THE ABILITY to undergo repeated deformation is a necessary requirement for RBC to pass through the microcirculation, and it is generally agreed that impairment of this ability results in blood flow disturbances. In sickle cell (HbSS) disease, the RBC have abnormal mechanical characteristics, and these abnormalities have been implicated in the pathological process.[1] This role of the RBC in the pathogenesis of HbSS disease has led many workers to study the rheological properties of both sickle cells and hemoglobin S (HbS) solutions. It has been demonstrated that, even when fully oxygenated, RBC suspensions and individual HbSS RBC exhibit abnormal rheological behavior: (1) sickle cell suspensions have impaired filterability[2,3] and elevated viscosity[4] as compared with normal RBC suspensions at equal hematocrit; (2) for dense HbSS RBC, cellular abnormalities include a more rigid cell membrane, an elevated internal viscosity, and a decreased overall cellular deformability.[5-9] These changes become accentuated when oxygen tension (ie, Po_2, partial pressure of oxygen) is lowered. At $Po_2 < 20$ mm Hg, sickled RSC (reversible sickle cells) have greatly reduced membrane and cellular deformability[10]. In addition, as Po_2 falls, the viscosity of HbSS RBC suspensions progressively increases and their filterability becomes further impaired[3,11,12]; these changes begin at Po_2 levels above those necessary to cause RBC sickling (ie, morphological transformation of cells to characteristic "holly leaf" or elongated forms with spicules). However, a small fraction of the intracellular HbS may be polymerized at these Po_2 levels; this polymer fraction appears to be insufficient to cause morphological changes but great enough to initiate flow disturbances.[13]

It appears most likely that the Po_2-dependent changes in sickle cell properties noted above arise from alterations in the physical state of the cytoplasmic HbS. This interrelation of cellular rheological behavior and cytoplasmic properties has been demonstrated by Chien et al[14] in studies of the viscoelastic behavior of sickle cell suspensions and of HbS solutions. Although suspensions of both oxygenated HbSS and normal RBC exhibit viscoelastic behavior (ie, have measurable viscous and elastic components of the complex viscosity), only sickle cell suspensions show increasing viscoelastic components with decreasing oxygen tension.[14] These changes in suspension viscoelasticity can be correlated with alterations in the cytoplasm of the HbSS RBC, in that cell-free HbS solutions have no elastic component when oxygenated but develop viscoelastic characteristics with decreasing Po_2. In fact, the viscous and elastic components become so great for HbS solutions that Chien et al[14] have suggested that the viscoelastic behavior of HbSS RBC suspensions at low oxygen saturations is dominated by the RBC cytoplasmic properties. The potential influence of cytoplasmic alterations on cellular behavior is also demonstrated by the finding that, at zero Po_2, HbS solutions form gels, exhibit "solid-like behavior," and have a threshold stress level (ie, a yield stress) for the initiation of flow.[15]

In contrast to studies of RBC suspensions and of HbS solutions, few data exist for the effects of Po_2 on the mechanical properties of individual HbSS RBC. Both the resistance to deforming forces and the rate of the deformation response are likely to change for HbSS RBC as Po_2 is decreased, and both these characteristics will influence RBC flow behavior in vivo. Although it is known that sickled cells are more resistant to deformation at reduced Po_2,[10] little is known of the dynamic aspects of their response. Nor is it known at what Po_2 these viscoelastic characteristics first become altered, how the alterations vary with Po_2, or

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whether alterations occur for cells that have not undergone morphological sickling. The present investigation was designed to explore these questions by carrying out micropipette aspiration studies of individual HbSS RBC over a wide range of oxygen tensions. For sickled HbSS cells, membrane tongues were aspirated into pipettes with diameters in the range of 1.2 to 1.6 μm; the rate and final extent of membrane deformation were measured. In a similar manner, the membrane shear elastic modulus (μ) (ie, membrane rigidity) was measured for normal HbAA cells and for HbSS RBC which remained in the normal discocytic shape. Discocytes were also subjected to extensional recovery measurements where, after micropipette elongation, the rates at which they regained their resting shapes were determined. Our aim was to interrelate changes in cytoplasmic state with alterations in cellular viscoelasticity and morphology, and to gain a better understanding of the role that RBC mechanical abnormalities may play in the pathogenesis of sickle cell disease.

MATERIALS AND METHODS

Donor population and RBC suspensions. Blood samples were obtained from a total of 21 patients with homozygous HbSS disease, with the approval of the University of Southern California Human Subjects Research Committee. Patients were defined by the usual hematologic criteria.[16] Excluded from the study were patients in sickle cell crisis and those who had received transfusions within the prior three months. All patients were seen at the Comprehensive Sickle Cell Center, Los Angeles County–University of Southern California Medical Center, Los Angeles. Control samples were obtained from healthy adult laboratory personnel. Blood was collected by venipuncture into heparin (5 IU/mL) and measurements were completed within 24 hours of withdrawal. All cell preparations and measurements were carried out at room temperature (23 ± 1 °C) unless otherwise specified.

Two different buffered saline solutions were used: phosphate-buffered saline (PBS, 0.122 mol/L of NaCl, 0.03 mol/L of KH_2PO_4 + Na_2HPO_4, 0.2g/dL of glucose, pH 7.4, 300 mosmole/kg) and bicarbonate-buffered Ringer solution (BRS, 0.128 mol/L of NaCl, 0.025 mol/L of $NaHCO_3$, 0.003 mol/L of KCl, 0.002 mol/L of $MgSO_4$, 0.0025 mol/L of $CaCl_2$, 0.1g/dL of glucose, equilibrated with 95% air/5% CO_2, pH 7.4, 300 mosmole/kg). To these buffers was added either 0.2g/dL of human serum albumin (HSA) or 9% vol/vol autologous plasma. Except where stated otherwise, RBC suspensions were made by diluting whole blood to a hematocrit of ~0.05% in these media.

Micropipette apparatus. The microscope-video and micropipette systems were as previously described.[8,17] The micropipette chambers used for the cell suspensions were modified to minimize air/sample contact and thus to decrease the rate at which Po_2 would rise for the suspensions with low oxygen tension. The chambers were fabricated from standard glass microscope slides and coverslips, separated by rectangular, 1-mm thick parafilm gaskets. The gaskets had narrow openings (5-mm) on one edge for pipette entry and the final size of the chambers was 27 × 15 mm. Micropipette manipulations of the RBC were carried out in the central part of the chamber, ~10 to 12 mm from the entrance. The pipettes were hydraulically coupled to a micrometer-driven hydrostatic reservoir for variation of the aspiration pressure (P). Because for some measurements relatively high P and rapid changes in P were desired, this system could be switched, by a valve, to a closed, fluid-filled system. The pressure in this closed system was varied using a screw-driven, gas-tight syringe (Hamilton Co, Reno, Nev), and was measured using a Validyne pressure transducer (model DP7, Validyne Engineering Corp, Northridge, Calif). The two pressure systems could be interconnected for zero pressure adjustment and for calibration purposes.

Variation of Po_2 and measurement of RBC viscoelasticity. Two types of studies were carried out at various Po_2 levels: (1) measurement of sickled, spiculed HbSS cells by observation of their time-dependent response to changes in micropipette aspiraton pressure; and (2) measurement of disc-shaped HbSS and control HbAA cells by micropipette determination of the membrane μ and the time constant (tc) for shape recovery. Different preparative and measurement procedures were used for these experiments.

Sickled HbSS RBC. HbSS RBC, suspended in 15 mL of BRS plus plasma, were placed in a 125-mL flask and orbitally shaken while a humidified 95% N_2/5% CO_2 gas mixture was blown over the liquid surface. After an interval (usually 5 to 30 minutes, depending on the Po_2 desired), a portion of the suspension was withdrawn into a syringe through narrow-bore plastic tubing. The Po_2 of the suspension in the syringe was measured, and a portion of the suspension from the same syringe was then introduced into the micropipette chamber, which had been previously flushed with humidified 95% N_2/5% CO_2. Following micropipette analysis (ie, after ~20 to 45 minutes), the suspension in the chamber was withdrawn, and its Po_2 was remeasured.

The viscoelastic behavior of sickled cells was tested by measuring the time-dependent response of membrane tongues which were aspirated into micropipettes with an internal radius (Rp) in the range of ~0.6 to 0.8 μm. A small initial suction pressure (eg, 3 mm H_2O) was applied to the micropipette, and its tip was placed in contact with a smooth region of the surface of a sickled cell, away from spicules or sharp edges (see Fig 1). This small initial pressure did not cause deformation of the sickled cells and served only to hold the cell to the pipette tip. The P was then rapidly increased (ie, in ~1 to 2 seconds) using the closed pressure system, and the membrane tongue growth was observed and video recorded. Depending on the Po_2 of the suspension, the pressures applied ranged up to 500 mm H_2O. After a period of time (usually ≳ 1 minute), P was either further increased or was reduced in steps. From the video recordings, tongue length was measured as a function of time during both the pressure increase (ie, loading) and decrease (ie, unloading) portions of the experiment.

Discocytic HbSS and HbAA RBC. During the above mentioned studies of sickled cells, it was noted that some HbSS RBC remained

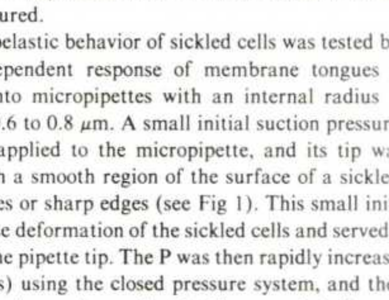

Fig 1. Photograph taken from a video recording of micropipette aspiration of a spiculed, sickled HbSS RBC. The Po_2 was in the range of 30 to 50 mm Hg, the pipette radius was 0.6 μm and the aspiration pressure was 30 mm H_2O; the resultant tongue length was approximately equal to two pipette radii (ie, L/Rp ~ 2). Note that the portion of the cell outside of the pipette was not distorted by the aspiration process.

in the normal, discocytic shape at all Po_2 tested. To study the mechanical properties of these discocytic HbSS cells, as well as of those of HbAA erythrocytes, a different preparative procedure was required. It was necessary to use cells which were point-attached to the upper surface of the micropipette chamber and to use buffer/HSA and buffer/plasma mixtures that had various fixed and predetermined Po_2 levels. In addition, it was necessary to manipulate these buffers and the micropipette chamber in an environment with a defined Po_2. To meet these requirements, gas mixtures containing desired proportions of N_2 and air were prepared using a dual flow-meter (Air Products Inc, Tamaqua, Pa), and all sample preparation and chamber loading procedures were carried out inside a glove box that was continuously purged with the gas mixture. Cells suspended in PBS plus HSA, and cell-free PBS plus plasma were equilibrated for 30 minutes with a gas mixture; a portion of the cell suspension was then loaded into a gas-flushed micropipette chamber. The chamber was then inverted for 10 to 20 minutes to allow cells to settle and attach to its upper surface. The PBS plus HSA was subsequently aspirated from the chamber and replaced with PBS plus plasma at the same Po_2; the presence of plasma in the medium inhibits further cell adhesion to glass surfaces during measurements of tc.[18] In this series of experiments, Po_2 was measured for the original cell suspension, for the original PBS plus plasma mixture and, after micropipette analysis, for the contents of the micropipette chamber.

Discocyte viscoelastic behavior was quantitated by measuring the tc for extensional shape recovery and the membrane μ. The membrane surface shear viscosity was calculated from these measured parameters by the relation, $\eta_m = \mu \cdot tc$.[18] The methods for measuring tc and μ were those previously described by Nash et al.[8] To determine tc, the length and width of a point-attached cell were measured at intervals after it had been elongated by micropipette manipulation and then released. The exponential shape recovery process was analyzed, and tc was calculated by the method of Hochmuth et al.[18] The membrane μ was measured by the micropipette method of Evans and LaCelle[19] for cells detached from the chamber surface. A membrane tongue was aspirated from the cell's flat surface into a micropipette of radius Rp (Rp = 0.6 to 0.8 μm) and the tongue length (L) was measured as the P was raised in steps. The rate of change of L with P was determined by linear regression and used to calculate μ, ie, $dL/dP = Rp^2/(2.5\mu)$; this is a linear approximation[20] to the exact theory of Evans.[21,22]

Miscellaneous techniques. The partial pressure of oxygen (Po_2) was measured using a Radiometer Model PH71 system equipped with an oxygen electrode (Radiometer Company, Copenhagen, Denmark). pH was measured by the same Radiometer system, and solution osmolalities were determined by freezing point depression (Model 2007, Precision Systems, Inc, Sudbury, Mass). The percentage of sickled cells present at various Po_2 levels was determined from microscopic observations of fixed cells. For these measurements, RBC suspensions of 1% to 2% hematocrit were either gassed for different time periods with 95% N_2/5% CO_2 or equilibrated with different N_2/air mixtures as described above. The fixative (2% glutaraldehyde in PBS) was gassed or equilibrated in the same manner and mixed at a ratio of 1:1 with the cell suspension. The Po_2 levels of both the original cell suspension and the cell/fixative mixture were measured, and the average was taken. For two donors, the percentage of cells sickled was determined at 37 °C as well as at 23 °C; these suspensions were equilibrated for 30 minutes with N_2/air mixtures of known Po_2. The Po_2 levels of the 37 °C suspensions were not measured, but were assumed to equal those measured for the room temperature suspensions which were concurrently and identically gassed. Sickled cells were categorized as those cells that had altered, angular morphology, usually with long spicules and often with an elongated shape. ISC were included in the total RBC count, but were not considered as sickled unless spiculated.

RESULTS

Data for the percentage of cells sickled *v* Po_2 are shown in Fig 2. At room temperature (23 °C), a lower Po_2 was required to initiate sickling than at 37 °C; for a Po_2 of 17 mm Hg, 50% of cells were sickled at 23 °C, whereas at 37 °C, 50% sickling occurred at a Po_2 of about 35 mm Hg. Unfixed cell preparations, observed in the micropipette chambers, also contained sickled cells if the sample initially had a Po_2 of $\leq$ 35 mm Hg. In these low Po_2 preparations, most ISC retained their oxygenated shape and had no spicules. However, under high magnification ($\times$10,000), video-microscopic observation, it was evident that at low Po_2 these nonspiculated ISC had developed roughened surfaces and a granular or stippled cytoplasm. Moreover, in these preparations, some approximately disc-shaped HbSS cells had a similar surface and cytoplasmic granularity. These nonspiculated ISC and granular disc-shaped cells had morphologies resembling those obtained when dense HbSS RBC fractions are deoxygenated[23] and were easly distinguishable from unaltered, smooth surfaced ISC and discocytes.

Sickled cells exhibited one of two distinct types of deformation response when subjected to micropipette aspiration of membrane tongues, ie, either an essentially elastic or a plastic response (Fig 3). For cells behaving elastically, application of P caused the growth of a tongue whose L increased slowly toward a final stable level. Subsequently, if P was raised further, a new stable length (Lf) tended to be achieved (Fig 3A, cell I). For these cells, if the P was returned to zero, the tongue relaxed without evident residual deformation; P could also be decreased in steps with a concurrent step-wise reduction in L (Fig 3A, cell II). Other cells, with an essentially plastic response, exhibited continuous membrane tongue growth, until a bud of membrane-bound material formed and broke away from the cell (ie, irreversible plastic membrane deformation, Fig 3B, cell I). In some cases,

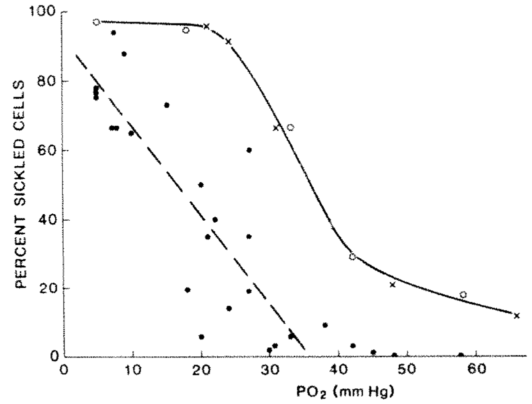

Fig 2. Percentage of sickled cells *v* Po_2 (mm Hg) for samples either at 23 °C ± 1 °C (- - - -) or 37 °C ± 0.5 °C (——). At 23 °C, samples from 12 donors were tested; the line shown was fitted by linear regression of the data for Po ≤40 mm Hg ($r = -.852$, $P < .001$). Two of the 12 samples were also tested at 37 °C (ie, symbols o and x), and the line shown was drawn by hand.

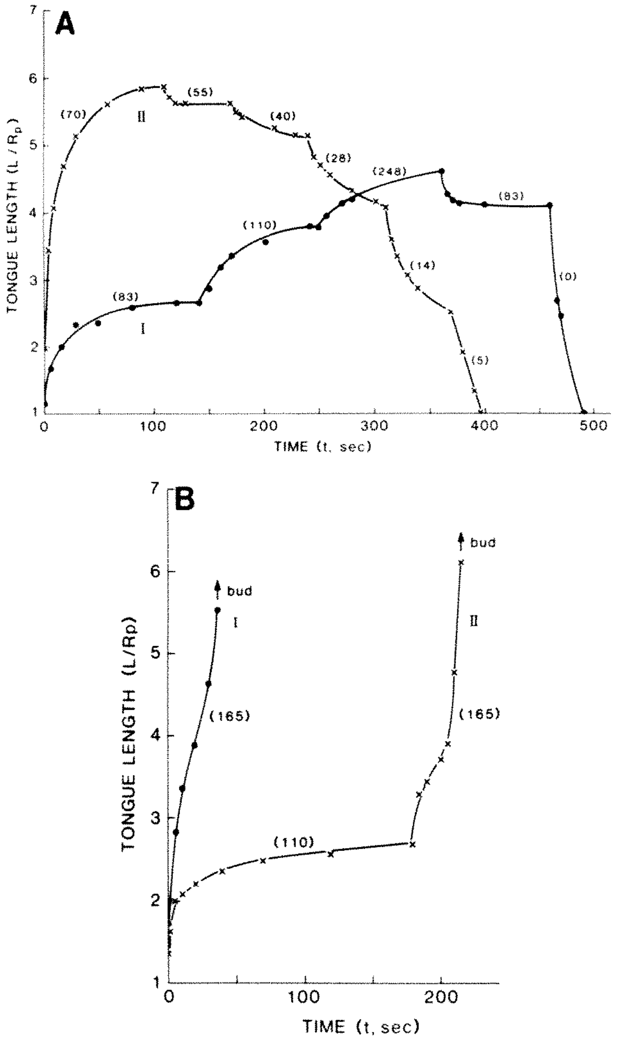

Fig 3. Aspirated membrane tongue length *v* time (seconds) for sickled cells; tongue length (L) is normalized by the pipette radius (Rp). The aspiration pressure (P, mm H_2O) is shown in brackets for each section of the curves. (A) Data for two cells behaving essentially elastically; for both cells Rp = 0.6 μm and Po_2 was in the range of 30 to 50 mm Hg. (B) Data for two cells exhibiting plastic deformation, with membrane buds formed for $L/Rp \gtrsim 6$. For cell I, Rp = 0.8 μm and Po_2 was in the range of 5 to 26 mm Hg; for cell II, Rp = 0.6 μm and Po_2 was in the range of 5 to 40 mm Hg.

residual deformation was seen if P was returned to zero before budding occurred, ie, some membrane remained protruding from the side of the cell. Such residual tongues were crumpled and flaccid, showing that the Hb originally inside the tongue had flowed back into the cell. For some cells, after stable deformation had occurred at one pressure, a subsequent increase in P led to rapid tongue growth and budding (Fig 3B, cell II). Plastic deformation was not analyzed in detail, other than to note that for this type of deformation, budding generally occurred for $L/Rp \gtrsim 6$, and that tongue growth was, on the average, initially several times faster than that of cells which responded elastically. Whenever possible, plastic deformation of cells was avoided; this was achieved by using pressures which were below those necessary to cause irreversible deformation. During either elastic or plastic deformation, the overall sickled cell shape was not perceptibly altered by the micropipette procedure.

Cells that behaved essentially elastically took ~30 to 120 seconds to form a membrane tongue with a stable length Lf. A measure of the rigidity of these cells was obtained by measuring this final Lf for each cell at 1 to 4 increasing pressures. Then an "effective membrane rigidity" (EMR) was calculated in a manner analogous to that used to determine the μ of discocytes. The quotient Lf/P was taken, or if more than one data pair existed, dLf/dP was calculated by linear regression. This value was multiplied by $(2.5/Rp^2)$ and the reciprocal of the product was taken as the EMR. This measure of rigidity (ie, EMR) has the same units as the membrane μ, but we do not imply that it reflects a true membrane property. Rather, EMR values were used to characterize the resistance to deformation of sickled cells at various Po_2 levels and to compare the deformation response of sickled cells and discocytes. Figure 4 shows EMR plotted *v* Po_2; the data demonstrate that the rigidity of sickled cells increases rapidly as Po_2 decreases. If EMR values are compared to the μ values previously obtained for oxygenated HbSS discocytes (mean = 6.7×10^{-3} dyne/cm, see ref 8), it is evident that all sickled cells are highly resistant to deformation. For example, at $Po_2 \sim 35$ mm Hg, the EMR of sickled cells was ~tenfold greater than the μ for discocytes; at $Po_2 \sim 10$ mm Hg, it was ~fifty-fold greater. If the line drawn in Fig 4 is extrapolated to predict the Po_2 at which the

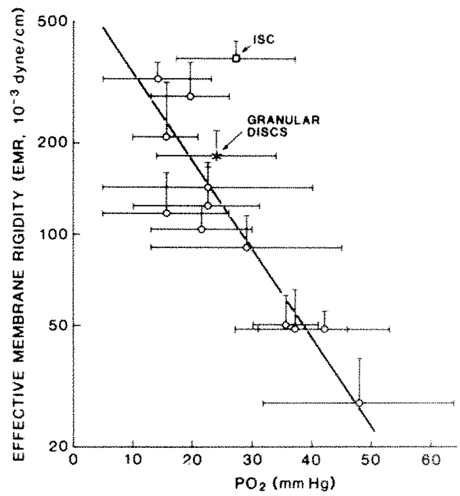

Fig 4. Effective membrane rigidity (EMR, 10^{-3} dynes/cm) *v* Po_2 (mm Hg) for sickled cells behaving essentially elastically. The horizontal bars indicate the range of Po_2 from that measured when the sample was loaded into the chamber to the higher value remeasured after micropipette analysis; all data points are located at the average of these two Po_2 measurements. For spiculed, sickled cells (O), the points represent the mean ± SEM of EMR values from 3 to 7 cells; ten donors were tested at one or two Po_2 ranges. The line drawn represents the linear regression of ln(EMR) against Po_2 ($r = -.955$, $P < .001$). For the nonspiculated, irregular ISC (☐) and the granular discs (•), the data were pooled from three donors tested at similar Po_2 ranges; a total of five ISC and eight discs were tested, and points represent the mean ± SEM.

EMR for sickled cells will equal the above mentioned rigidity (ie, μ) for oxygenated HbSS discocytes, a value of 70 mm Hg is found. However, since at 23 °C sickled cells do not exist above a Po_2 of ~40 to 50 mm Hg (see Fig 2), it seems likely that a step increase in rigidity occurs at the same time as morphological sickling. EMR values for ISC and for discocytes, both with irregular, granulated surfaces, are also shown in Fig 4. These cells had similar or slightly greater rigidity than the spiculed, sickled cells at comparable Po_2 levels.

For sickled cells that behaved elastically, data for tongue L *v* time (eg, Fig 3) were used to carry out an analysis of the rate of the deformation response. When P was first applied, an initial, short membrane tongue (~1 μm) was evident within a few seconds (ie, only slightly longer than the time required to raise P). L then slowly increased towards its final value (Lf). The initial, ~1 μm of tongue growth was not resolvable due to optical limitations, although the time course of the later growth could be analyzed. These length *v* time data did not follow a simple exponential approach toward Lf, and thus a single exponential tc could not be fitted to the data. Instead, half-times ($t^1\!/\!_2$) were used to characterize the rates at which sickled cells responded to deforming forces. This $t^1\!/\!_2$ was taken as the time required for L to increase from its initial value, when a tongue was first optically measurable (Li), to a value of (Li + Lf)/2, ie, halfway to its final length Lf. Figure 5 shows $t^1\!/\!_2$ values plotted as a function of Po_2; the values are based on membrane tongue growth resulting from the first applied pressure step. Half-times varied between ~5 to 35 seconds and were longer for lower Po_2. There was no significant correlation of $t^1\!/\!_2$ with P for a given sample, although it was observed that a longer half-time was usually obtained for the second pressure step. Note that the half-times shown in Fig 5 are approximately two orders of magnitude longer than either the time required for establishment of membrane tongues when normal RBC are aspirated into micropipettes[24] or the time required for recovery of cell shape after micropipette elongation of RSC.[8]

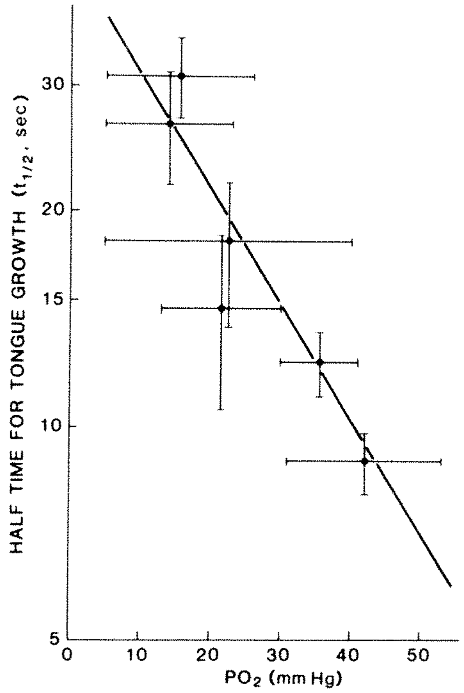

Fig 5. Half-time for membrane tongue growth ($t^1\!/\!_2$, seconds) *v* Po_2 (mm Hg) for sickled cells behaving essentially elastically. The horizontal bars indicate the range of Po_2 from that measured when the sample was loaded into the chamber to the higher value remeasured after micropipette analysis; all data points are located at the average of these two Po_2 measurements. Points represent the mean ± SEM for 3 to 5 cells from each of six donors. The line drawn represents the linear regression of ln ($t^1\!/\!_2$) against Po_2 ($r = -.932$, $P < .01$).

The horizontal bars shown in Figs 4 and 5 represent the increase in Po_2 that occurred between the initial loading of the sample into the micropipette chamber and the final remeasurement of its oxygen tension. However, it is likely that the cells which were actually measured experienced less variation in Po_2 than is indicated by these horizontal bars. When the sample was removed for final Po_2 measurement, fluid from the chamber entry region mixed with fluid from further within the chamber, where the pipette measurements were carried out. We expect the Po_2 to be higher for the outer than for the inner fluid, and thus the final measured Po_2 to be higher than the level experienced by any measured cell. If the horizontal bars in Figs 4 and 5 are indeed overestimates, the true EMR *v* Po_2 and $t^1\!/\!_2$ *v* Po_2 curves may lie slightly to the left (ie, lower Po_2) of the lines drawn, since the points shown are placed at the averages of the measured initial and final Po_2 levels.

Two other abnormalities were noted in the deformation response of sickled cells; both are illustrated in Fig 6, which shows typical data for stable tongue Lf *v* P. First, there was a large degree of hysteresis in the pressure-deformation response when P was reduced; ie, in Fig 6, the Lf *v* P data do not follow the same path for increasing and decreasing P.

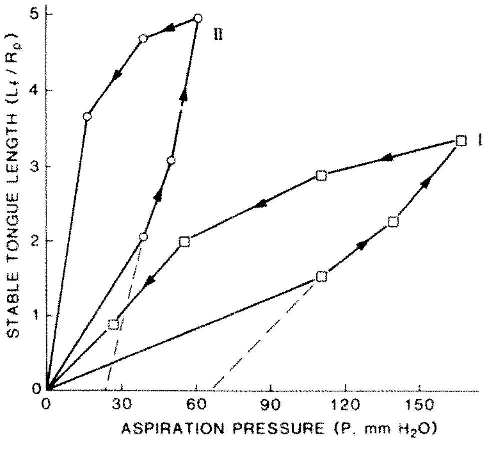

Fig 6. Stable membrane tongue length (Lf/Rp) *v* aspiration pressure (P, mm H_2O), for two sickled cells behaving essentially elastically. For cell I, Rp = 0.8 μm and Po_2 was in the range of 5 to 26 mm Hg; for cell II, Rp = 0.6 μm and Po_2 was in the range of 32 to 64 mm Hg. The arrows on the solid lines indicate the sequence of pressure changes, and the dashed lines represent linear regression extrapolations of the loading phase data to Lf/Rp = 0. Note that these dashed lines have a positive P intercept, thus indicating a resistance to initial tongue formation.

Greater deformation was observed at a given P during the unloading phase than was measured at that P during loading. This hysteresis tended to be more notable at lower Po_2 and exceeded that previously observed during deformation of oxygenated, dense HbSS RBC.[8,9] The presence of hysteresis indicates that, even for the cells whose behavior we have termed "essentially elastic," the deformation response deviates from that of a true elastic material. Second, it was noted that if P was initially raised from zero in small steps, a large P was often required before membrane deformation became apparent. This large resistance to initial tongue growth is evidenced by the non-zero intercepts of the dashed lines shown in Fig 6; these lines represent extrapolation of the Lf *v* P data for the loading phase to Lf = 0. The non-zero intercepts indicate that after an initial higher resistance to deformation was overcome, tongue growth subsequently occurred more easily. Such a phenomenon could represent the existence of a yield shear stress for HbS gel deformation, the presence of strain in the resting cell membrane induced by its distortion during sickling, or the resistance of HbS polymers to initial bending. Thus, the abnormal rigidity of sickled cells could represent contributions from several mechanical abnormalities.

Smooth HbSS discocytes with unaltered morphology were present at all Po_2 levels used in the present study, although at the lowest oxygen tensions, they were only present in small numbers; in the micropipette chamber, they could be seen adjacent to sickled cells. Data for the viscoelastic characteristics of these discocytic cells are shown as functions of Po_2 in Fig 7. There was no significant correlation of either the μ or the tc for shape recovery with Po_2. All the sample means obtained for these two mechanical properties at reduced Po_2 fell within the ranges previously found for oxygenated HbSS discocyte samples.[8] Also shown in Fig 7 are data for normal HbAA RBC; these control cells were measured for Po_2 in the range of 5 to 25 mm Hg and at Po_2 = 150 mm Hg. The μ and tc values measured for these HbAA RBC at reduced Po_2 were not significantly different from those for cells equilibrated at 150 mm Hg. Thus, no changes in membrane elasticity or membrane viscosity (ie, $\eta_m = \mu \cdot \text{tc}$) were detected for HbSS RBC that maintained discocytic morphology, or for HbAA cells. Consequently, neither the resistance to deformation nor the rate of the deformation response would vary with Po_2 for these discocytic cells.

DISCUSSION

The results presented herein indicate that when morphological sickling occurs, cellular deformability becomes greatly impaired and that this impairment increases as Po_2 is lowered. Our EMR data are in general agreement with the estimate of decreased membrane deformability obtained by LaCelle[10] for sickled cells at $Po_2 < 20$ mm Hg, although the large changes in ISC rigidity noted here (Fig 4) were not detected in that study. Moreover, we have found that the response of sickled cells to deforming forces is extremely slow. These alterations in mechanical behavior parallel the changes that have been noted previously in the viscoelasticity of sickle cell suspensions and of HbS solutions as Po_2 is reduced.[14] Hence, it seems likely that the deformation response of an individual sickled cell is dominated by the viscoelastic properties of its cytoplasm.

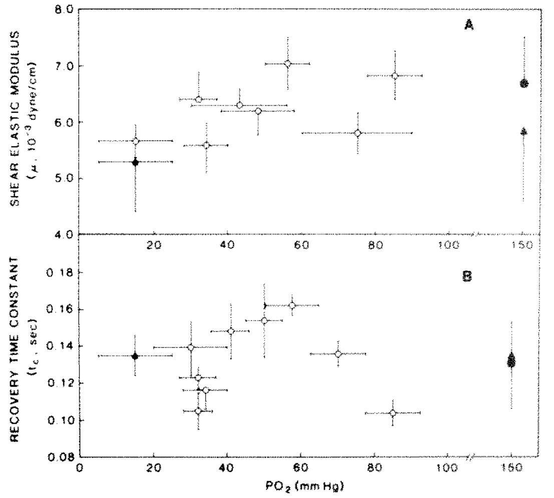

Fig 7. (A) Shear elastic modulus (μ, 10^{-3} dynes/cm), and (B) time constant for extensional shape recovery (tc, seconds) *v* Po_2 (mm Hg) for HbSS and HbAA discocytes. The horizontal bars indicate the range of Po_2 from that measured when the sample was loaded into the chamber to the higher value remeasured after micropipette analysis; all data points are located at the average of these two Po_2 measurements. Data for HbSS discocytes at $Po_2 <$ 100 mm Hg (○) are mean ± SEM for an average of 7 cells. In panel A, samples from eight donors were tested, each at a single Po_2 range; in panel B, samples from five donors were tested at one or more Po_2 ranges. Data are also shown for HbSS discocytes at Po_2 = 150 mm Hg (●) (ie, equilibrated with room air; from Nash et al.[8]) The points represent the mean ± SD of sample means from ten donors. For normal HbAA RBC (▲), points are mean ± SD of sample means from three donors. Micropipette tests were carried out at Po_2 = 150 mm Hg and in the range of 5 to 25 mm Hg for each HbAA donor. Note that for both the μ and the tc for shape recovery, neither HbSS nor HbAA RBC showed significant variations with Po_2.

As oxygen tension is reduced, formation of a sufficient amount of HbS polymer could cause the development of a new cellular elastic-resistive element and a very large increase in cytoplasmic viscosity, thus causing the cellular changes measured in the present study. The viscoelastic solid nature of this HbS polymer, and its entry into the micropipette during the deformation studies, is evidenced by the following experimental observations: (1) For cells behaving elastically (Fig 3A), membrane tongues reached a Lf rather than continuing to grow, although the aspiration pressures were too high to be supported solely by the unaltered membrane[8]; (2) in these elastic cases, deformation was recoverable when the pressure was released (Fig 6); (3) rates of tongue deformation were too slow to be the result of the flow of only the Hb solution phase into the pipette; (4) for cells showing plastic deformation (Fig 3B), the HbS within the tongue returned to the main portion of the cell if the pressure was released before budding occurred, although the membrane itself was irreversibly deformed. A nonelastic Hb solution would not return to the main portion of the cell, since there would be no driving force to cause this flow.

The above considerations suggest that the cellular deformation response will depend on the fraction of the HbS that is polymerized. In the Appendix, we describe calculations to determine polymer fraction (Fp) at 23 °C, for cells containing different HbS concentrations. The calculated plots of Fp *v* Po_2 are shown in Fig 8; superimposed are our experimental data for EMR *v* Po_2 (Fig 4) and for percentage of cells sickled *v* Po_2 (Fig 2). It can be seen that elevated rigidity occurs in the region where polymer is predicted to exist, and that EMR increases with Fp. However, there does not appear to be a simple, linear relation between EMR and Fp; there is no theoretical reason to suppose that Fp, cytoplasmic viscoelasticity, and cellular rigidity can be simply and linearly interrelated. Sickling itself appears to start at a Po_2 at which only the most dense cells [ie, hemoglobin concentration (Ct) $\gtrsim$ 40 g/dL] would contain polymer (Fig 8), but all cells are predicted to contain some polymer at the Po_2 at which 50% of the cells were observed to have sickled.

Discocytes that do not undergo sickling at a given reduced Po_2 may still contain some polymerized HbS, and it has been suggested that these cells will have impaired flow properties.[13] Indeed, changes in HbSS RBC suspension viscosity and filterability begin at oxygen tension levels higher than those at which sickling would be expected.[3,11,12] These flow abnormalities may be caused by changes in cytoplasmic viscosity which are not sufficient to affect micropipette measurements, since all discocytes measured in the present study exhibited normal rigidity and shape recovery rates. Deficiencies in cellular deformation response were detected only for those cells that had undergone changes in shape (ie, sickled forms) or surface appearance (ie, granular discs or ISC). Whether a cell sickles at a given Po_2 is likely to depend on its total Ct and/or on its content of fetal hemoglobin (HbF), since either low Ct or high HbF inhibits polymer formation.[12,25] HbF levels were measured for the donors tested in the present study and fell within the range of 0.2% to 13.1%. Based on the findings of Dover et al,[26] this range would mean that between 0.5% to 34% of the RBC would be so-called F cells; these cells contain disproportionately high levels of HbF, averaging 38% of their Ct.[26] At the lowest Po_2 levels, at which few discocytes were present, those discocytes that were subjected to micropipette analysis could preferentially be F cells. However, the discocytes tested at higher Po_2 could not all be F cells, since the percentage of discocytes exceeds the calculated proportion of F cells (see Fig 2). Whatever the mechanisms are that protect some cells from sickling, our data can be interpreted to show that enough polymer must form inside a cell to cause morphological sickling before the cell's deformability is grossly impaired.

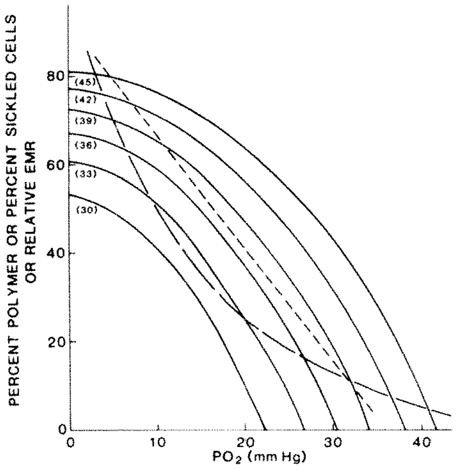

Fig 8. Calculated polymer fraction (Fp, in percentages) *v* Po_2 for HBSS RBC at 23 °C (——). Figures in brackets are the hemoglobin concentrations (Ct, g/dL) for each curve. Also shown are the lines previously fitted to the experimental data for percentages of sickled cells *v* Po_2 in Fig 2 (- - - -) and for relative effective membrane rigidity *v* Po_2 in Fig 4 (— —). Note that the relative EMR data (ie, EMR/μ) were calculated using a shear elastic modulus for oxygenated HbSS RBC (μ) taken from ref.[8]

Variations in cellular Hb concentration within samples and between donors could contribute to the experimental variability found in EMR and t½ values within individual RBC samples (ie, vertical bars in Figs 4 and 5) and between different donors tested at similar Po_2 levels. In addition to polymer formation, the viscosity of deoxygenated HbS solutions[14] and the elastic modulus of HbS gels[27] are strongly dependent on Hb concentration. Those sickled cells that deformed continuously and underwent plastic deformation may represent cells that had lower Ct and hence less severely impaired deformability. The present investigation is somewhat limited; although different cell types could readily be distinguished on the basis of their morphology or deformation response, the structural bases for these differences are not definitely known. Thus, the role of Hb concentration in determining variations in cellular deformation response at reduced Po_2 deserves further experimental studies. For example, density fractionation could be carried out prior to deformability analyses. Such a prolonged set of measurements may be more feasible if a simplified test procedure is used, eg, measuring the pressure required for total entry of cells into larger 3-μm pipettes.[10]

In contrast to the possible protective influence of low Hb concentration on the development of mechanical abnormalities, dense cells with high Hb concentration could be particularly significant in the pathogenesis of HbSS disease. These dense cells are probably the first to develop polymer-induced abnormalities as Po_2 is reduced. This suggestion regarding the possible detrimental effect of dense cells is supported by the results of the present study, in that: (1) cells with irregular surfaces (ISC and granular HbSS discocytes), which were found at reduced Po_2 levels, resembled cells found in deoxygenated dense cell fractions by Kaul et al[23]; (2) these irregular cells were present at oxygen tensions at which few spiculed, sickled cells were observed and tended to be more rigid than spiculed cells tested at comparable Po_2 levels (Fig 4).

It is not certain how closely the Po_2-dependent changes which we have measured at 23 °C reflect the changes that occur for HbSS RBC in vivo. We found that sickled cells with impaired deformability existed at $Po_2 \lesssim 40$ mm Hg. However, at 37 °C, sickling occurs at higher Po_2 levels (Fig 2). Because mixed venous blood typically has $Po_2 \sim 40$ mm Hg, and lower levels exist in some regions of the circulation, sickling probably occurs in vivo. Changes in mechanical properties, comparable to those measured in the present study, would make it highly likely that these sickled cells would obstruct narrow blood vessels.[28] Along with increased cell rigidity and decreased rates of deformation, the existence of elongated and irregular spiculed shapes could contribute to vascular occlusion. The mechanical abnormalities detected in the present study are much more severe than those we have previously found in dense oxygenated ISC and some RSC.[8] Thus, sickling may be a potentially more important cause of vascular occlusion than is the existence of some poorly deformable dense oxygenated cells, although the latter have been suggested to play a role in initiating the occlusive process.[4,28,29]

ACKNOWLEDGMENT

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APPENDIX

The fraction of polymerized hemoglobin (Fp) inside an HbSS RBC which contains a total hemoglobin concentration (Ct) is given by: $Fp = Cp \cdot (Ct - Cs)/Ct \cdot (Cp - Cs)$, where Cs is the HbS solubility and Cp is the polymer concentration of 69 g/dL.[30] Sunshine et al[31] have measured the solubility of HbS at 23 °C for various solution phase oxygen saturations (Ysol). Using the above equation and their data for Cs, Fp was calculated as a function of Ysol. To determine the variation of polymer fraction with Po_2, the relation between Ysol and Po_2 at 23 °C was required; this is not the same as the oxygen saturation of HbSS cells, since cells can contain a mixture of HbS solution and polymer. However, oxygen saturation *v* Po_2 data for normal HbAA blood can be used to represent the oxygen affinity of HbS in the solution phase (ie, Ysol *v* Po_2), since HbS and HbA have the same oxygen affinity when in solution.[32] Using oxygen affinity data for HbAA RBC at 23 °C,[33] polymer fraction *v* Po_2 relations were calculated for various total cellular hemoglobin concentrations (ie, Ct) at 23 °C. The predicted Fp *v* Po_2 curves are shown in Fig 8 for a chosen set of Ct. They indicate, for example, that at 23 °C, HbS polymer formation starts at a Po_2 of ~35 mm Hg for cells with Ct $\gtrsim$ 39 g/dL, and at about 20 mm Hg for cells with Ct $\lesssim$ 30 g/dL.

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High Molecular Weight Kininogen: Localization in the Unstimulated and Activated Platelet and Activation by a Platelet Calpain(s)

By Alvin H. Schmaier, Paulette M. Smith, A. David Purdon, James G. White, and Robert W. Colman

High mol wt kininogen (HMWK), the major cofactor-substrate of the contact phase of coagulation, is contained within and secreted by platelets. Studies have been performed to localize platelet HMWK in both the unstimulated and activated platelet and to ascertain the effect of platelet enzymes on HMWK itself. On platelet subcellular fractionation, platelet HMWK was localized to α-granules, and platelets from a patient with a deficiency of these granules (gray platelet syndrome) had 28% normal platelet HMWK. Platelet HMWK, in addition to being secreted from the platelet, was also localized to the surface of the platelet when activated. Using a competitive enzyme-linked immunosorbent assay for HMWK as an indirect antibody consumption assay, the external membrane of thrombin-activated platelets as well as the releasate from these stimulated platelets had 17 ng HMWK antigen/10^8 platelets available, whereas unstimulated platelets and their supernatant had only 4.9 and 4.2 ng HMWK/10^8 platelets present, respectively. The anti-HMWK antibody consumption by activated normal platelets was specific for membrane-expressed platelet HMWK, since activated platelets from a patient with total kininogen deficiency did not adsorb the anti-HMWK antibody. Enzymes in the cytosolic fraction of platelets cleaved ^{125}I-HMWK (mol wt 120,000) into a mol wt 100,000 polypeptide as well as smaller products at mol wt 74,000, mol wt 62,000, mol wt 47,000, and a few components below mol wt 45,000. No cleavage products were observed when DFP and leupeptin were present. The cleavage of HMWK was specifically prevented by inhibitors of calcium-activated cysteine proteases (leupeptin, *N*-ethylmaleimide, iodoacetamide, and EDTA) but not by inhibitors of serine proteases (DFP, benzamidine, soybean trypsin inhibitor, or aprotinin). Platelet cytosol increased the coagulant activity of exogenous purified HMWK with maximum HMWK coagulant activity (35-fold) occurring within ten minutes of exposure to platelet cytosol. Treatment of platelet cytosol with leupeptin prevented the increase in the coagulant activity of exogenous HMWK. These studies indicate that activated platelets express platelet HMWK on their external membrane and platelet enzymes can cleave and increase the coagulant activity of exogenous HMWK.

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HIGH MOL WT kininogen (HMWK) is the major cofactor for contact phase zymogen (factor XII, prekallikrein, factor XI) activation as well as their major substrate. Recent studies suggest that plasma HMWK may be a procofactor which, when cleaved, may elaborate higher surface-mediated cofactor activity. Bovine intact HMWK, kinin-free kininogen, and a large fragment consisting of fragment 1 · 2 and light chain accelerate the generation of factor XII and kallikrein activity on a kaolin surface, whereas kinin and fragment 1 · 2 free protein, heavy chain, or low mol wt kininogen do not.[1] Further studies show that kallikrein-cleaved bovine HMWK, preceding kinin liberation, accelerates kaolin-mediated activation of bovine factor XII over intact HMWK.[2] The accelerating effect of cleaved bovine HMWK on factor XII activation is mediated through its adsorption on the kaolin surface through the fragment 1 · 2 region and its complex formation with prekallikrein through the light chain region.[3] More recent studies with human HMWK indicate that the amount of HMWK adsorbed to kaolin is closely correlated with its coagulant activity, and the ability of HMWK to adsorb to kaolin increased with time of kallikrein digestion to a maximum, paralleling bradykinin liberation.[4] These studies suggest that HMWK is a procofactor whose cleavage by kallikrein activates the molecule, allowing enhanced coagulant activity and bradykinin liberation.[4]

Our finding that platelets contain HMWK, a fourth cofactor in plasma coagulation reactions which is secreted after platelet activation,[5] suggest that platelets may provide an in vivo activating surface. To characterize the ways in which platelet HMWK and platelets themselves may contribute to contact phase activation, a study was initiated to determine the location of platelet HMWK in unstimulated and activated platelets and to determine whether platelet enzymes alter HMWK. This work shows that platelet HMWK is a platelet α granule protein in the unstimulated platelet that is expressed on the activated platelet membrane after thrombin stimulation. Moreover, platelets contain a calcium-activated cysteine protease [Calpain(s)] which can cleave the procofactor HMWK and, concommitant with this cleavage, increase its coagulant activity.

MATERIALS AND METHODS

Materials. Antiserum to fibrinogen was purchased from Atlantic Antibodies, Scarborough, Me. Nagarse was purchased from the Enzyme Development Corp, New York. Human α thrombin (3209 NIH U/mg) was a kind gift of Dr J. Fenton, New York State Department of Health, Albany, NY. Na ^{125}I (50 mCi/mmol) was obtained from New England Nuclear, Boston. Iodogen (chloroamide, 1,3,4,6-tetrachloro-3 α-6 α-diphenyl glycoluril) was obtained

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from Pierce Chemical Co, Rockford, Ill. Standards for mol wt determination on polyacrylamide gels in sodium dodecyl sulfate (SDS) were purchased from BioRad Corp, Richmond, Calif. Antisera to bradykinin was a generous gift of Dr David Proud, Johns Hopkins University, Baltimore, Md. All other reagents were obtained from Sigma Chemical Corp, St Louis, Mo.

Plasma and Platelets. Pooled normal plasma (lot No. 120 and N10) was purchased from George King Biomedical, Inc, Overland Park, Kan. These lots of plasma were previously determined to contain 80 and 88 μg/mL, respectively, of HMWK antigen by comparison of the plasma antigen to purified HMWK reconstituted into total kininogen-deficient plasma.[5] HMWK antigen was assayed by electroimmunodiffusion using monospecific antisera to the light chain of HMWK.[5] Total kininogen-deficient (ie, deficient in both HMWK and low mol wt kininogen) plasma[6] and platelets[5] were donated by Mrs M. Williams, Philadelphia, Pa. Platelets from a patient (R.K.) with the gray platelet syndrome[7] was directly donated to this laboratory. This patient had a plasma HMWK level of 63 μg/mL as determined by electroimmunodiffusion.[5] Normal donors, who provided written informed consent, were young men and women (ages 21 to 40) on no medication. Fresh blood was collected, and platelet-rich plasma and platelet-poor plasma were prepared as previously described.[8] In the experiments with the platelet calcium-activated protease (Calpain), supernatants of lysed platelets refers to a 12,000 g supernatant of frozen and thawed platelets. In these same experiments, cytosols of lysed platelets refers to a 100,000 g supernatant of frozen and thawed platelets. Elsewhere in the text, use of the term supernatant of platelets simply refers to the soluble material obtained after centrifugation.

Preparation of HMWK. HMWK was purified from fresh plasma using a modified procedure of Kerbiriou and Griffin[9] that included 0.2 mol/L of ϵ amino caproic acid in all buffers and 2 mmol/L of diisopropylfluorophosphate (DFP) added to the pooled HMWK prior to each step in the purification procedure. ϵ Amino caproic acid was added to the buffers of the purification to prevent the possibility of plasmin proteolysis of the HMWK during the purification procedure.[10] This preparation of HMWK, reduced on 7.5% polyacrylamide gel electrophoresis (PAGE) with SDS,[11] was a single band of mol wt 120,000. The specific activity of the purified HMWK varied from 12 to 20 U/mg. HMWK coagulant activity was measured by a one-stage kaolin-activated assay using total kininogen-deficient plasma as substrate[6] as described previously.[5] In brief, samples were compared against a daily standard curve from pooled normal human plasma diluted in 0.01 mol/L of Tris-HCl, 0.15 mol/L of NaCl, pH 7.4. The final dilutions of plasma ranged from 1/10 to 1/1,000 and were linear on a log–log plot. One unit was defined as the amount in 1 mL of pooled normal plasma.

Purified HMWK was radiolabeled with $Na^{125}I$ using Iodogen by the method of Fraker and Speck[12] as previously reported.[5] The radiolabeled protein was >95% trichloroacetic acid precipitable and retained >95% of its procoagulant activity as well as its antigenic properties. The specific radioactivity of the preparations of ^{125}I-HMWK used in these studies varied from 1 to 5 μCi/μg. Protein determinations were performed by the method of Bradford[13] using crystalline bovine serum albumin as the standard.

Anti-HMWK Antisera. Goat antisera directed against the light chain of HMWK were prepared from total kininogen antisera by successive adsorption with purified low mol wt kininogen as previously reported.[5,14] The monospecific antisera to the light chain of HMWK was used in a competitive enzyme-linked immunosorbent assay (CELISA).[5]

Localization of Platelet HMWK in the Unstimulated Platelet. Platelet subcellular fractionation was performed according to the technique of Fukami et al.[15] In each preparation, platelet-rich plasma from 450 mL of blood was collected, adjusted to contain 5 mmol/L of EDTA and pelleted by centrifugation at 3,000 g for ten minutes at 4 °C. The platelet pellet was suspended in 30 mmol/L of Hepes, 5 mmol/L EDTA and 0.15 mol/L of NaCl, pH 6.5. After being washed by two additional centrifugations at 3,000 g for ten minutes at 4 °C, the pellet was resuspended in 40 mL of the same medium containing 20 μmol/L of rotenone, 5 mmol/L of 2-deoxy-D-glucose and 10 mmol/L of D-gluconic acid δ lactone and was incubated at 37 °C for 20 minutes. Then, 3 mg of nagarse and 20 mg of ATP were mixed into the suspension at 37 °C. Five minutes later, 20 mg of soybean typsin inhibitor was added to neutralize the nagarse, and the platelets were centrifuged at 3,000 g for ten minutes at 4 °C. The pellet was then resuspended into 25 mL of 0.01 mol/L of Hepes, 1 mmol/L of EDTA, and 0.25 mol/L of sucrose, pH 7.4, and was homogenized twice in a French pressure cell[16] at 500 lb/sq in with one centrifugation in between so that only the unbroken cells were subjected to the second homogenization. Five milliliters of the combined homogenate was set aside, and 20 mL were used for fractionation. Differential centrifugation of the homogenate resulted in the isolation of four fractions: the F_1, which consists of unbroken platelets and large fragments, is a 1,000 g × 22-minute pellet; F_2, which contains platelet granules and mitochondria, is a 12,000 g × 20-minute pellet; F_3, which contains platelet membranes, is a 100,000 g × 60-minute pellet; and F_4, which is platelet cytosol, is a 100,000 g supernate. The granule fraction, F_2, was then divided in half and one half was applied to a 0.8 to 2.0 mol/L sucrose discontinuous step gradient in 0.2 mol/L increments.[17] After centrifugation at 100,000 g for 60 minutes, five distinct zones were collected: zone A, 0.25 to 1.0 mol/L of sucrose; zone B, 1.0 to 1.3 mol/L of sucrose; zone C/D, 1.3 to 1.8 mol/L of sucrose; zone E, 1.8 to 2.0 mol/L of sucrose; and zone F, the pellet. Radiolabeled HMWK tracer experiments were performed to determine that the measured HMWK in the granule fractions arose from those fractions and was not merely soluble plasma HMWK attached to or trapped in the granule material. Prior to assay, fractions F_4 and A through E of the F_2 were dialyzed against 0.01 mol/L of Hepes, 1 mmol/L of EDTA, pH 7.4, lyophilized, and resuspended in 5 mL of the dialysis buffer. Fractions were treated with 0.5% Triton X-100 prior to assay. HMWK antigen was assayed by CELISA using antisera directed to the light chain of HMWK.[5] The CELISA for HMWK used to determine the amount of HMWK in the total and each fraction of the subcellular fractionation of platelets was performed identically as previously described.[5] A standard competitive inhibition curve was obtained with normal plasma and plotted by a computer-fitted four-parameter logistic function.[18] The amount of HMWK in each part of the subcellular fractionation was obtained from the calculated slope of a linear regression generated by multiple dilutions of the unknown sample.[18] β-*N*-acetylglucosaminidase was assayed by the technique of Day et al.[19] Fibrinogen was assayed by electroimmunodiffusion.[20] Platelet serotonin was assayed by the technique of Weissbach et al.[21] Only preparations that had a recovery >60% for total protein, HMWK, fibrinogen, β-*N*-acetylglucosaminidase, and serotonin were used in the analysis. The "relative specific amounts" of each marker in the preparations were calculated from the ratio of the "specific amount" of the marker in the fraction (amount of antigen or activity per milligram of total protein) (F_1 through F_4; A through P) to the specific amount of the marker in the total platelet homogenate or F_2 fraction, respectively. The percentage of distribution of each protein in each fraction was determined by the ratio of the area under the bar graph for each fraction (relative specific amount times total protein) to the area of the sum of all fractions in either the differential centrifugation or sucrose density ultracentrifugation.

Studies on the total content of platelet HMWK in gray platelets and a concomitant normal control were done on blood anticoagulated with 3.8 g/dL sodium citrate containing PGE_1 (final concentration 1 μmol/L). After the platelet-rich plasma was separated,[8] washed platelets were obtained by the combined techniques of albumin density gradient centrifugation and gel filtration with buffer containing 1 μmol/L of PGE_1 performed as previously reported.[5] PGE_1 was added to the anticoagulant and gel filtration buffer to prevent loss of platelet HMWK during the washing procedure. The washed platelets were solubilized by the addition of Triton X-100 (0.5% final concentration) at room temperature for 30 minutes. The total platelet HMWK in these samples was assayed by CELISA performed identically as was previously reported.[5]

Localization of platelet HMWK in the Activated Platelet. Studies were performed to determine if platelet HMWK was detectable on the surface of activated platelets. Four hundred fifty milliliters of fresh blood from two donors was collected into 73 mmol/L of citric acid, 3 mmol/L of trisodium citrate containing 2% dextrose (1:10) (ACD), and platelet-rich plasma was obtained.[8] After the pH was adjusted to 6.5 with 1 mol/L of citric acid, the platelet-rich plasma was washed twice by the centrifugation technique of Mustard.[22] The Tyrode's buffer containing Ca^{2+} (2 mmol/L) and Mg^{2+} (1 mmol/L) had both apyrase and heparin (12.5 U/mL) in the first wash and apyrase alone in the second wash. Apyrase was prepared from potatoes by the method of Molnar and Lorand[23] and was titered so that the minimal amount necessary to prevent second-wave platelet aggregation with ADP was used. The final washed platelets were resuspended in Hepes buffer[24] to a total volume of 10.5 mL. In four experiments, the platelet concentration ranged from 1.75 to 8.8×10^9 platelets per milliliter. These platelet preparations contained $<0.05\%$ red cells and $<0.03\%$ leukocytes (monocytes, granulocytes and lymphocytes, combined). The washed platelets were then divided into three aliquots, each of 3.5 mL. Two aliquots received PGE_1 (final concentration 1 μmol/L) and were incubated for 40 minutes at 37 °C. The third aliquot was made to 0.4 mmol/L with Gly-Pro-Arg-Pro in order to prevent fibrin polymerization and platelet aggregation of activated platelets[25] and was placed in a flat-bottomed polystyrene tube in a 37 °C water bath. Thrombin (0.5 U/mL) was introduced, and the reaction was allowed to proceed for ten minutes without stirring. Immediately after the ten-minute incubation with thrombin, the contents of the tube were layered on a 2.5-ml discontinuous albumin density gradient (10%, 15%, 20%, 30%, 40%) prepared as previously reported.[5] After centrifugation at 2,000 rpm for 25 minutes at 23 °C in a GLC-2B centrifuge (Dupont Instruments, Wilmington, Del), 3.5 mL of activated platelet supernatant was separated from the activated platelets, which formed a layer over the 40% albumin cushion. The platelets were resuspended in 3.5 mL of Hepes buffer containing 0.4 mmol/L of Gly-Pro-Arg-Pro. A second platelet aliquot was centrifuged for four minutes at 12,000 g in an Eppendorf centrifuge (Brinkman Instruments, Inc, Waterbury, Conn), and the supernatant was collected. Thus, four specimens were collected: an aliquot of intact, unstimulated platelets, supernatant from unstimulated platelets, thrombin-activated platelet supernatant, and thrombin-activated platelets.

The platelet samples were prepared for use in a modified CELISA for HMWK as an indirect antibody consumption assay. Because membrane-expressed platelet HMWK would not be soluble to allow a direct determination of its presence, and solubilization of activated platelets would liberate intracellular platelet HMWK, an experimental design was developed to determine if surface-expressed platelet HMWK could be detected when whole platelets were incubated with the antisera directed to HMWK. Conditions for this antibody consumption assay for surface-expressed platelet HMWK were developed so that intraplatelet HMWK (that which was neither secreted nor expressed on the external membrane) would not interfere. The four platelet specimens were mixed with an equal volume of monospecific anti-HMWK antisera (final concentration 1:500). This antisera had previously been diluted 1:250 in 0.01 mol/L of sodium phosphate pH 7.4, 0.15 mol/L of NaCl containing 0.05% Tween-20 (PBS-Tween), and was also made 1:250 with total kininogen-deficient plasma. Before the prepared antisera was mixed with the platelet material, the antisera was ultracentrifuged at 100,000 g for 90 minutes in a Beckman Model L3-50 ultracentrifuge (Beckman Instruments, Inc, Palo Alto, Calif) to remove any aggregates that might be present. The total kininogen-deficient plasma was added to the incubation mixture to prevent nonspecific association of goat anti-HMWK IgG to human platelet Fc receptors by the addition of excess human IgG. After the four platelet samples were combined with the antisera, each mixture was incubated for one hour at 37 °C. In preliminary experiments, a one-hour incubation of purified HMWK or HMWK in normal plasma was sufficient to achieve $>$ a 90% interaction of soluble antigen and antibody when compared to a two- to three-hour incubation at 37 °C. At completion of the incubation, each specimen was centrifuged at 12,000 g in an Eppendorf centrifuge, and the supernatants were collected. Aliquots for lactic dehydrogenase determination[26] were taken from each of the four supernatant specimens to be compared to the amount of lactate dehydrogenase lost by solubilization of intact platelets by addition of 0.5% Triton X-100.

Four antibody samples per experiment were then compared by a CELISA assay for HMWK[5] using known amounts of purified HMWK antigen to determine the slope of the competitive inhibition curve produced by each batch of platelet-adsorbed or platelet supernatant-adsorbed antisera. The competitive inhibition curves generated on the CELISA by the four aliquots of adsorbed antisera were produced by the method of incubation of samples as previously reported.[5] However, since the aim of the assay was to determine whether the titer of the antisera would be decreased (consumed) by incubation with platelets or their supernatants, the final competitive inhibition curves were analyzed by nonlinear regression to determine the slope of the competitive inhibition curve produced by antisera adsorbed with each of the aliquots of platelet material. In all experiments, the differently adsorbed aliquots of antisera were reacted with the same amounts of purified HMWK (1 to 125 ng). The data were plotted as the concentration of the purified HMWK used to determine the slope of the competitive inhibition curve of the adsorbed antisera on the abscissa *v* the optical density at 405 nm on the ordinate. The measured value of this assay was the slope of the competitive inhibition curve. Statistical comparison of the differences of the slopes of the competitive inhibition curves was performed by grouped paired T test.

Quantification of the amount of platelet HMWK expressed on the surface of the platelet was obtained by comparing the values of the slopes produced by each sample of platelet-adsorbed or platelet supernatant-adsorbed antisera with a standard curve of the anti-HMWK antisera adsorbed with known concentrations of purified HMWK. In these latter experiments, the anti-HMWK antisera was was adsorbed by purified HMWK at various concentrations (20 to 2,000 ng/mL) for one hour at 37 °C. After incubation, the adsorbed antisera was interacted with purified HMWK to determine the adsorbed antisera's competitive inhibition curve. These slopes were also calculated by nonlinear regression. The data from these latter experiments were plotted as concentration of purified HMWK used to adsorb the antisera on the abscissa *v* the slope of the resultant competitive inhibtion curve on the ordinate. The amount of HMWK (plasma or platelet) associated with the surface of the platelet or in its suspending medium was estimated by comparing the slopes of the competitive inhibition curve produced by the antisera adsorbed with the platelet material with the slopes of the competitive inhibition curves produced by known concentrations of purified HMWK. The final results were calculated based on the number of platelets in each experiment and were expressed as nanogram of HMWK per 10^8 platelets.

Platelet proteolysis and activation of purified HMWK. The effect of supernatants and cytosols of platelet lysates on purified exogenous ^{125}I-HMWK and nonradiolabeled HMWK was studied. Platelets from 450 mL of blood were washed by the centrifugation technique of Mustard,[22] and the final washed pellet was resuspended in 1 to 2 mL of Hepes buffer[24] without bovine serum albumin and dextrose, or in 0.1 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4 (TBS) with or without 1 mmol/L of EDTA. After the platelet suspension was made hypotonic by addition of 0.5 to 1 mL of deionized water, the platelets were lysed by freezing and thawing four times on dry ice and at 37 °C. The supernatants of the lysed platelets were collected after centrifugation at 12,000 g in an Eppendorf centrifuge. In certain experiments, the supernatant of the platelet lysate was ultracentrifuged at 100,000 g for 90 minutes at 4 °C to collect a platelet cytosol. Supernatant and cytosol of lysed platelets were used to study the effect of platelet enzymes on ^{125}I-HMWK and exogenous HMWK. In certain experiments, the platelet lysate or cytosol was preincubated with cysteine or serine protease inhibitors for 10 to 30 minutes prior to the addition of the HMWKs. In experiments in which the platelet cytosol was prepared in the presence of 1 mmol/L of EDTA, the reaction was begun by the addition of 2.5 mmol/L of $CaCl_2$. All incubation mixtures of the platelet material and the HMWKs were performed at 37 °C. Determination of cleavage of exogenous ^{125}I-HMWK by platelets was performed by reducing a 25 to 50-μL aliquot of the platelet supernatant–cytosol–^{125}I-HMWK mixture with 2% β mercaptoethanol and adding it to a 7.5% polyacrylamide gel with SDS for electrophoresis. Detection of changes in the ^{125}I-HMWK was made by performing autoradiography on the dried gel. Determination of changes in activity of exogenous purified HMWK was made by coagulant assay[5] of the HMWK-platelet supernatants or cytosol mixtures. Bradykinin release from exogenous HMWK added to platelet cytosol was measured by radioimmunoassay performed as previously reported.[4]

RESULTS

Localization of platelet HMWK in the unstimulated platelet. Platelet subcellular fractionation studies were performed to determine the platelet location of platelet HMWK in the resting platelet. Six aliquots of washed platelets were evaluated for the subcellular localization of platelet HMWK by differential centrifugation (Fig 1). In these six preparations, the recovery (mean ± SEM) of total protein was 76% ± 3.2%; of HMWK antigen, 92% ± 9.5%; of fibrinogen antigen, 79% ± 14%; and of β-*N*-acetylglucosaminidase activity, 88% ± 5.8%. Radiolabeled HMWK tracer studies for the differential centrifugation separation were performed by the introduction of the ligand into the platelet homogenate after platelet lysis by the French Press. In these studies, 95.5% of the tracer was recovered in the F_4 cytosol fraction, 3.8% of the tracer was found in the F_3 membrane fraction, 0.4% of the tracer was found in the F_1 fraction, and only 0.3% of the tracer was localized to the F_2 granule/mitochondria fraction. When washed, lysed platelets were subjected to differential centrifugation, the largest amount (46% of the total antigen), as indicated by the area under the bar graph, of platelet HMWK antigen was found in the 12,000 g pellet (fraction F_2) (Fig 1). This localization of platelet HMWK to the F_2 fraction, a fraction previously characterized as enriched in platelet granules and mitochondria,[16] was similar to the subcellular localization found for two known platelet granule constitutents, fibrinogen and β-*N*-acetylglucosaminidase.

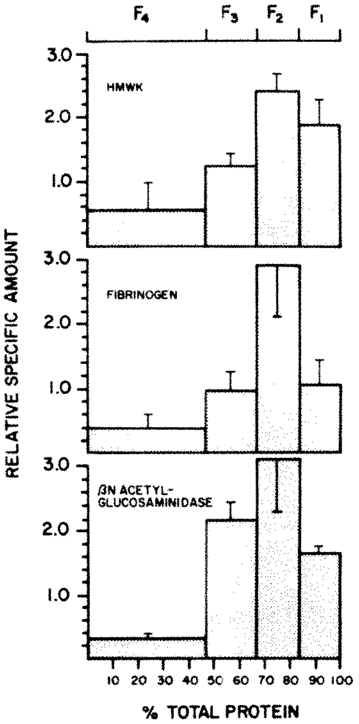

Fig 1. Platelet subcellular fractionation by differential centrifugation. Ordinate, the relative specific amount of each marker; abscissa, percentage of total protein in each fraction. The relative specific amount is the ratio of the specific amount in each fraction compared with the specific amount in the total homogenate of the platelets. The specific amount in a fraction or total is calculated by the ratio of the total antigen or activity in a fraction divided by the total protein in the fraction. The data plotted represent the mean ± SEM and the SEM is represented by the bars (T) of six experiments. F1, 1,000 g pellet; F2, 12,000 g pellet; F3, 100,000 g pellet; and F4, 100,000 g supernatant.

In an attempt to separate the components of the granule fraction further, the F_2 was subjected to sucrose density ultracentrifugation (Fig 2). In six experiments, the recovery (mean ± SEM) of total protein was 88% ± 2.6%; of HMWK antigen, 92% ± 7.8%; of fibrinogen antigen, 96% ± 10.5%; of β-*N*-acetylglucosaminidase, 80% ± 2.5%; and 98% ± 4.5% of serotonin. Radiolabeled HMWK tracer studies were also performed for the sucrose density gradient centrifugation separation with the presence of the ligand in the F_2 fraction. In these studies, 68% of the tracer remained in the soluble A fraction, 24% of the tracer was found in the C/D fraction, 4% of the tracer was found in the B fraction, 2.4% of the tracer was in the E fraction, and 1.5% of the tracer was trapped in the P fraction. Most platelet HMWK antigen entered the gradient (ie, was localized to fractions B through P, which indicated that it was in granules), and was found to be present in the largest amount, as indicated by the area under the bar graph, in the C/D and E fractions (Fig 2), fractions enriched with platelet α-granules.[15,17] The C/D fraction

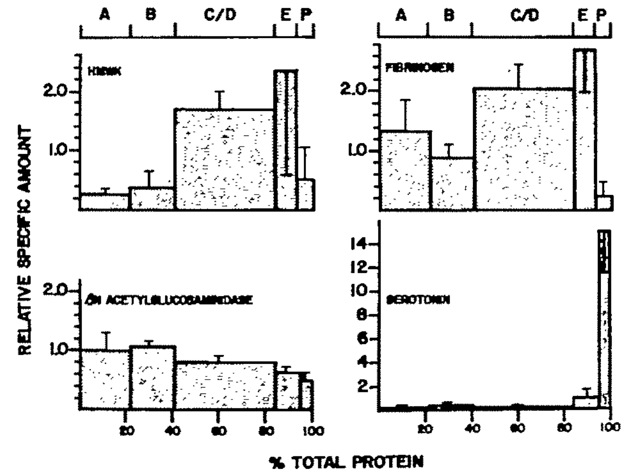

Fig 2. Further fractionation of F2 from Fig 1 by sucrose density ultracentrifugation. The data plotted represent the mean SEM and the SEM is represented by the bars (T) of six experiments. All data are plotted as in Fig 1. A, supernatant to 1 mol/L of sucrose; B, 1 to 1.3 mol/L of sucrose; C/D: 1.3 to 1.8 mol/L of sucrose; E: 1.8 to 2.0 mol/L of sucrose; F, the 100,000 g pellet of the gradient.

accounted for 65% of the total platelet HMWK, but only 44% of the total protein found in the F_2 fraction. Although 24% of the soluble ^{125}I-HMWK tracer was found in the C/D fraction, the amount of trapped soluble HMWK in this fraction was really only 0.072% of the total HMWK measured since the F_2 fraction only contained 0.3% of the soluble HMWK. This finding by the radiolabeled tracer studies indicated that most of the HMWK antigen measured in the C/D fraction must have been derived from platelet granules. This localization of platelet HMWK was similar to that of platelet fibrinogen, a known platelet α-granule constituent.[27,28] β-*N*-acetylglucosaminidase, a lysosomal granule marker enzyme, was spread throughout the gradient, and serotonin, a dense granule constituent, was concentrated in the 100,000 g pellet (P). These combined studies suggested that platelet HMWK was mostly a platelet α granule protein in the unstimulated platelet.

To confirm independently that most platelet HMWK in the unstimulated platelet was localized to platelet α-granules, platelets from a patient (R.K.) with the gray platelet syndrome[7] were studied for their total content of platelet HMWK. These platelets contained 16 ng/10^8 platelets total platelet HMWK, whereas a concomitant normal platelet control contained 52 ng/10^8 platelets. Comparing the value of total platelet HMWK in gray platelets with the normal control and nine previously determined values[5] for total platelet HMWK in normal platelets (60 ng ± 24/10^8 platelets), the gray platelets studied contained only 28% of the content of platelet HMWK.

Localization of platelet HMWK in the activated platelet. Studies were initiated to determine whether platelet HMWK became expressed on the external platelet membrane after activation. Platelets, in an unstimulated or activated state, along with their respective suspension buffers, were compared using a modified CELISA for HMWK antigen as an indirect antibody consumption assay. Anti-HMWK antisera was adsorbed with unstimulated or activated platelets and their supernatants to determine whether the titer of free anti-HMWK antibody (initial dilution 1:500) would be decreased. The detection of a decrease in the titer of the specific antibody was determined by a change in the slope of a competitive inhibition curve produced by the adsorbed antisera from each platelet aliquot when subsequently reacted with the same amounts of purified HMWK. In preliminary experiments, competitive inhibition curves produced by increasing dilution (1/500, 1/800, 1/1,100, and 1/1,500) of anti-HMWK antisera incubated with identical amounts of purified HMWK resulted in a flattening (decrease in the slope) and a shift to the left of the competitive inhibition curve as the dilution of anti-HMWK antisera was increased from 1/500 to 1/1,500 (data not shown). The relationship between the change in the calculated slope of the competitive inhibition curve produced by anti-HMWK antisera adsorbed with exposed HMWK antigen was studied in a quantitative manner (Fig 3). Anti-HMWK antisera initially

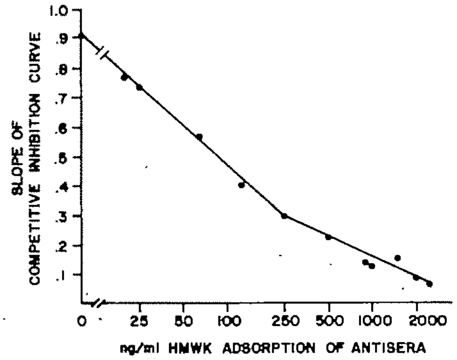

Fig 3. The relationship of the slope of the competitive inhibition curve produced by anti-HMWK antisera *v* the concentration of HMWK used to adsorb the antisera. Anti-HMWK antisera was prediluted 1:500 in PBS-Tween and incubated for one hour at 37 °C in the absence or presence of purified HMWK at a concentration from 20 to 2,000 ng/mL in the suspending buffer. At the conclusion of the 1-hour incubation, the adsorbed antisera was incubated overnight at 37 °C with an equal volume of purified HMWK in an absolute amount from 1 to 125 ng. The next day, the contents of the above incubations were added to radioimmunoassay-grade bovine serum albumin-coated (0.2%) polystrene cuvette wells that had been linked with 150 ng of purified HMWK in 0.1 mol/L of sodium carbonate, pH 9.6. After two-hour incubation and after the cuvettes were washed with PBS-Tween, the free anti-HMWK antibody, which had not been previously consumed by the two incubations with soluble HMWK and which had bound to the HMWK linked to the cuvette well, was exposed to a rabbit anti-goat antibody conjugated with alkaline phosphatase. After a further two-hour incubation, the amount of anti-HMWK antibody bound to the cuvette wells was detected by the addition of 0.05 mol/L of sodium carbonate, pH 9.8, containing 1 mmol/L of MgCl and 1 mg/mL *p*-nitrophenylphosphate disodium. The hydrolysis of the substrate was stopped at 30 minutes by the addition of an equal volume of 2 mol/L of NaOH to the volume already contained in the cuvette wells. The inhibition curves produced by the unadsorbed and adsorbed antisera were calculated by nonlinear regression. The calculated slope of the adsorbed antisera was plotted on the ordinate and the concentration of purified HMWK used to adsorb the antisera for one hour was plotted on the abscissa. Each point represents the mean of two or three experiments.

diluted 1:500 produced a competitive inhibition curve with a calculated slope by nonlinear regression of 0.9. Incubating the anti-HMWK antisera initially diluted 1:500 in separate aliquots with increasing concentrations of purified HMWK (20 to 2,000 ng/mL) for one hour at 37 °C resulted in competitive inhibition curves with reduced calculated slopes (0.77 to 0.06) (Fig 3). This study indicated that the slope of a competitive inhibition curve produced by antisera adsorbed under the identical conditions could be used to calculate the amount of exposed HMWK antigen indirectly.

Unstimulated and activated platelets and their supernatants were incubated with anti-HMWK antisera initially diluted 1:500. In one representative experiment (Fig 4), the competitive inhibition curves produced by antisera adsorbed with the supernatant of unstimulated platelets (slope 0.34) and unstimulated platelets (slope 0.33) themselves gave parallel and almost superimposable curves as previously reported.[5] The competitive inhibition curve produced by antisera adsorbed with the activated platelet supernatant was flattened with a decreased slope (0.20) when compared with that produced by the supernatant of unstimulated platelets (Fig 4). Because platelet HMWK is secreted by thrombin-activated platelets,[5] this finding indicated that secreted platelet HMWK adsorbed and decreased the titer of the anti-HMWK antibody. The competitive inhibition curve produced by activated platelets (slope 0.26) was similar to the curve that characterized the material released by platelets (Fig 4). Antisera adsorbed with activated platelets showed a competitive inhibition curve that had a reduced slope and was left-shifted when compared with unstimulated platelets. This adsorption of the antisera by activated platelets could not be owing to platelet HMWK lost from cell lysis, because the amount of lactic dehydrogenase loss under the conditions of the experiments was $<5\%$. This result suggested that some of the platelet HMWK, in addition to being secreted, must also have been expressed on the external membrane of the activated platelet.

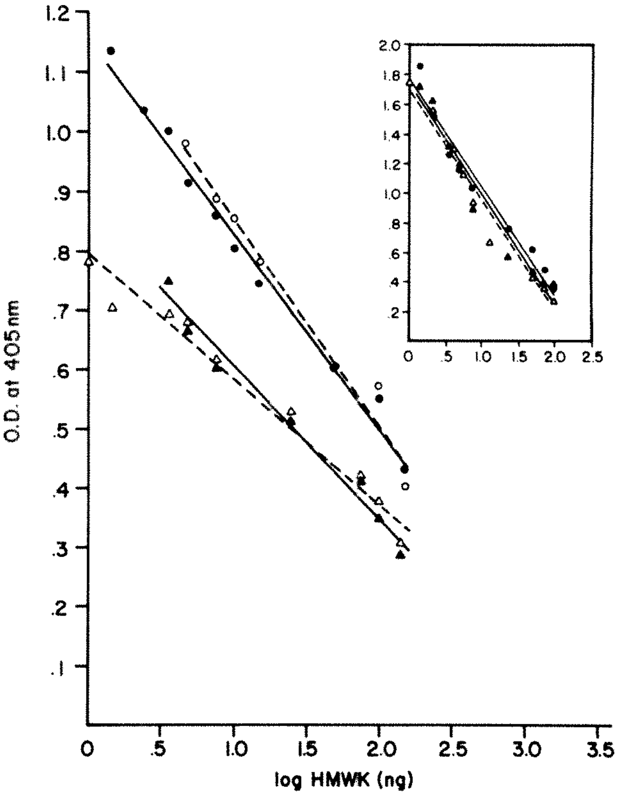

Fig 4. Expression of platelet HMWK antigen on the activated platelet surface. The competitive inhibition curves produced by anti-HMWK antisera adsorbed with unstimulated platelet supernatant (O---O), unstimulated platelets (●——●), activated platelet supernatant (Δ---Δ), and activated platelets (▲——▲) are plotted. Ordinate, observed optical density (OD) at 405 nm; abscissa, absolute amount (log HMWK in ng) of purified HMWK incubated with the prepared antisera. One representative experiment of four with normal platelets is depicted. Inset: competitive inhibition curves produced by anti-HMWK antibody consumed by total kininogen-deficient platelets. The competition inhibition curves produced by anti-HMWK antisera adsorbed with unstimulated (●——●) and activated (▲---▲) total kininogen-deficient platelets as well as the activated platelet supernatant of total kininogen-deficient platelets (Δ---Δ) are plotted. All data in the inset are plotted as above. In both plots, the competitive inhibition curves were calculated by nonlinear regression.

In four experiments, the slopes of the competitive inhibition curves produced by each of the four platelet aliquots in each experiment were calculated by nonlinear regression. Anti-HMWK antisera adsorbed by unstimulated platelets and unstimulated platelet supernatant produced competitive inhibition curves with calculated slopes (mean ± SEM) of 0.37 ± .015 and 0.37 ± .011, respectively. The slopes produced by antisera adsorbed by unstimulated platelets and supernatants from unstimulated platelets in each experiment were not significantly different ($P > .45$). Anti-HMWK antisera adsorbed by activated platelets and activated platelet supernatant produced competitive inhibition curves with calculated slopes (mean ± SEM) of 0.22 ± .027 and 0.21 ± .017, respectively. The slope produced by antisera adsorbed by activated platelets and their supernatants was not significantly different ($P > .4$). However, the slopes produced by supernatants from activated platelets and activated platelets themselves were significantly decreased ($P < .0125$, $P < .005$, respectively) when compared to unstimulated platelets. This finding indicated that both specimens had adsorbed the antibody with expressed platelet HMWK. Because the slope of the competitive inhibition curve produced by adsorbed antisera is directly related to the concentration of HMWK available to consume the specific antibody (Fig 3), an estimate of the amount of HMWK associated with each of the platelet aliquots was calculated based on the number of platelets in each experiment. In the four experiments, unstimulated platelets and their supernatant had a mean of 4.9 ng HMWK/10^8 platelets and 4.2 ng HMWK/10^8 platelets, respectively, associated with the material. Alternatively, activated platelets and their supernatant had a mean of 17.3 ng HMWK/10^8 platelets and 17.2 ng HMWK/10^8 platelets, respectively, associated with the aliquots. The increased amount of HMWK made available from the activated platelet specimens must have been derived from the intraplatelet pool of HMWK. The similarity in the decrease in the slopes in the competitive inhibition curves and the calculated amount of available platelet HMWK between the activated platelets and their supernatant indicated that the amount of platelet HMWK expressed on the external platelet membrane after platelet activation is similar to the amount of platelet HMWK secreted from activated platelets.

It is possible that the antibody consumption by thrombin-activated normal platelets was not specifically due to expressed platelet HMWK, but rather due to alterations on the platelet surface as result of thrombin stimulation. In order to evaluate this possibility, the competitive inhibition curves produced by antibody adsorbed by unstimulated and thrombin-activated total kininogen-deficient platelets,[5] as well as the activated deficient platelet supernatant, were compared (inset, Fig 4). Adsorption of the anti-HMWK antisera by unstimulated total kininogen-deficient platelets, thrombin-activated total kininogen-deficient platelets, and activated deficient platelet supernatant produced competitive inhibition curves with calculated slopes of 0.71, 0.78, and 0.77, respectively. These slopes were similar to the competitive inhibition curve produced by unadsorbed antisera and, when corrected for the number of platelets in the experiment, gave values for available HMWK <1 ng HMWK/10^8 platelets—the lower limit of sensitivity of the assay. These data indicated that the consumption of the anti-HMWK antibody by activated platelets was specific for the surface expression of platelet HMWK.

Platelet proteolysis of purified ^{125}I-HMWK. Studies were initiated to determine if platelet enzymes have any effect on the structure of purified HMWK. Initial studies were performed on supernatants of frozen and thawed platelets in the presence or absence of DFP (5 mmol/l) and leupeptin (0.5 mmol/L). Platelets were lysed by freezing and thawing as described in the Materials and Methods Section. As shown in Fig 5 (right), uninhibited supernatants of platelet lysates (2.1×10^{10} platelets per milliliter) produced increasing extent of proteolysis of exogenous purified ^{125}I-HMWK as a function of the lysate concentration. The initial proteolysis of the untreated samples, seen best with platelet lysate concentrations of 10% and 20%, resulted in the appearance of an intermediate mol wt 100,000 band, accentuation of a mol wt 75,000 band which existed in trace amounts in the starting material, a thick mol wt 62,000 band, and the appearance of a mol wt 47,000 band along with small cleavage products at the dye front. As the concentration of platelet lysate increased (40% to 100%), the starting mol wt 120,000 band and intermediate mol wt 100,000 band disappeared, the thick mol wt 62,000 band increased in intensity, and a few new bands, below mol wt 47,000, appeared. Combined treatment of supernatants of platelet lysates with 5 mmol/L of DFP and 0.5 mmol/L of leupeptin prevented all proteolysis (Fig 5, left).

Nature of the Platelet Protease(s) That Cleaves ^{125}I-HMWK. The nature of the platelet protease(s) that produced cleavage of ^{125}I-HMWK was studied further. Platelet cytosol made from 1.0×10^{10} platelets per milliliter was prepared and, in individual aliquots, was pretreated with a wide variety of serine and cysteine protease inhibitors (Fig 6). The addition of leupeptin (0.5 mmol/L), iodoacetamide (0.5 mmol/L), *N*-ethylmaleimide (0.5 mmol/L) and EDTA (10 mmol/L) prevented the proteolysis of ^{125}I-HMWK into a mol wt 100,000 band as well as multiple bands of lower mol wt by platelet cytosol. However, pretreatment of platelet cytosol with DFP (5 mmol/L), soybean trypsin inhibitor (100 μg/mL), benzaminidine (5 mmol/L), or aprotinin (1,000 KIU), did not. This finding indicated that a divalent cation dependent cysteine protease(s) [Calpain(s)] from the platelet cytosol was mostly responsible for cleavage of exogenous ^{125}I-HMWK into a mol wt 100,000 band along with the appearance of other lower mol wt bands.

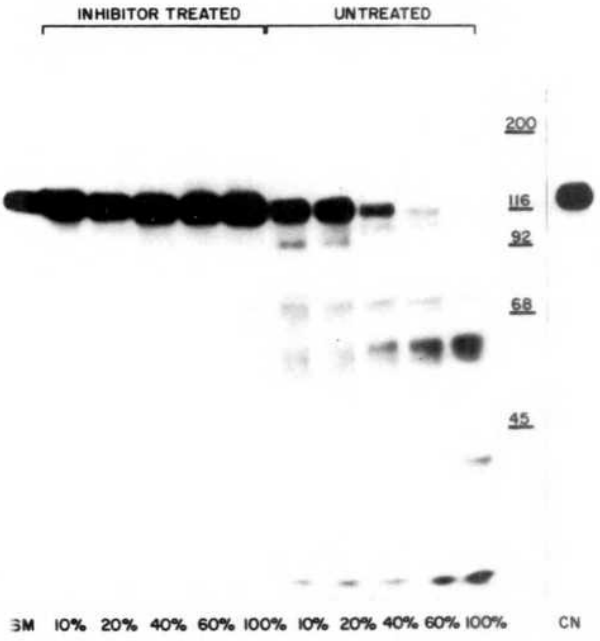

Fig 5. Platelet proteolysis of purified ^{125}I-HMWK. Platelets were washed and resuspended in 3 mL of wash buffer and deionized water (2:1) and were lysed by freezing and in thawing; the supernatant from 2.1×10^{10} platelets per milliliter was divided in half. One-half of the supernatant was incubated for 30 minutes at room temperature in the presence or absence of DFP (5 mmol/L) plus leupeptin (0.5 mmol/L). After incubation, five 100-μl samples were made of both the inhibitor-treated and untreated samples consisting of 10%, 20%, 40%, 60%, or 100% of platelet lysate in (TBS) 0.1 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4. At the conclusion of the incubation, ^{125}I-HMWK (1.25 nmol/L) (specific radioactivity 3.3 μCi/μg) was added to each aliquot, and the mixture was incubated for ten minutes at 37 °C. At the conclusion, all samples were frozen at −70 °C. For the gel, 25 μL from each aliquot was reduced with 2% β-mercaptoethanol and applied directly to a 7.5% polyacrylamide gel in sodium dodecyl sulfate for electrophoresis; after drying, autoradiography was performed. SM represents 3.75 ng of the ^{125}I-HMWK starting material applied directly to the gel. CN represents an incubation in which purified ^{125}I-HMWK (1.25 nmol/L) was added to platelet buffer and handled identically to the platelet samples. The number to the right of the gel represents mol wt standards.

Activation of HMWK by a platelet protease. Although a platelet divalent cation dependent cysteine protease was responsible for cleavage of HMWK, the significance of this cleavage was unclear. To study whether changes in the HMWK molecule altered its activity, purified HMWK in a final concentration of 3 U/mL (12.7 U/mg) was added to platelet supernatants pretreated with leupeptin or left untreated (Table 1). As seen in Table 1, platelet lysate alone

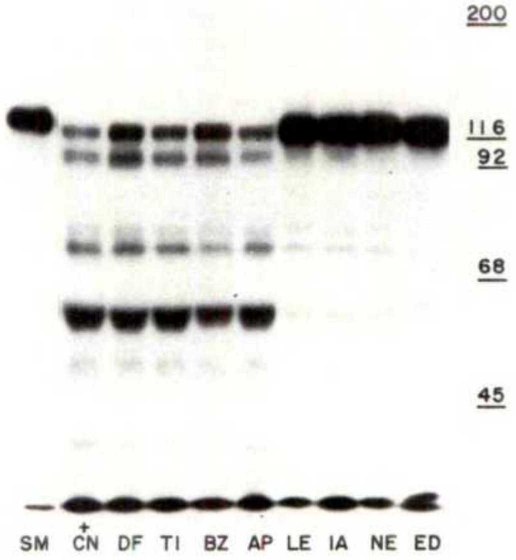

Fig 6. Effect of protease inhibitors on the appearance of the 100,000 mol wt cleavage product of ^{125}I-HMWK. A 100,000 g cytosol of platelets (1.0 × 10^{10} platelets per milliliter) were treated with various serine and cysteine protease inhibitors for ten minutes at 37 °C. Thirty nanograms of ^{125}I-HMWK (5 nmol/L with a specific radioactivity, 3 μCi/μg) was added to an 80% mixture of inhibitor-treated platelet cytosol and incubated for one hour at 37 °C. SM, ^{125}I-HMWK incubated in buffer; (+)CN, ^{125}I-HMWK added to a mixture which consisted of 80% platelet cytosol in 0.1 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4 (TBS). DF, ^{125}I-HMWK added to platelet cytosol pretreated with 5 mmol/L of diisopropylfluorophosphate (DFP); TI, 4.76 nmol/L of soybean trypsin inhibitor; BZ, 5 mmol/L of benzamidine; AP, 1,000 KIU of aprotinin; LE, 0.5 mmol/L of leupeptin; IA, 0.5 mmol/L of iodoacetamide; NE, 0.5 mmol/L of *N*-ethylmaleimide; and ED, 10 mmol/L of EDTA. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate and auto radiography were performed as described in Fig 5. The numbers to the right of the gel represent mol wt standards.

contributed little coagulant activity (<0.1 U/mL) and leupeptin in the concentration used (0.5 mmol/L) did not inhibit the coagulant assay, since leupeptin-treated buffer (2.5 U/mL) had similar coagulant activity to untreated buffer (2.9 U/mL). However, when comparing the coagulant activity of the exogenous HMWK in the leupeptin-treated (3.1 U/mL) *v* the untreated specimen (22 U/mL, 93.6 U/mg) after a two-hour incubation, HMWK exposed to the untreated platelet supernatant exhibited sevenfold increased specific activity. Because the purified HMWK was added in a final concentration of about 3 U/mL, the increased activity measured in the untreated specimen must have been due to "activation" of the exogenous HMWK by a leupeptin-sensitive protease. In separate experiments not shown in Table 1, leupeptin in a concentration of 0.1 mmol/L and iodoacetamide (0.5 mmol/L) also inhibited the increase in exogenous HMWK coagulant activity by platelet cytosols.

Table 1. Platelet Activation of HMWK

Material*	(+) Leupeptin† (U/mL)	(−) Leupeptin (U/mL)
Platelet lysate + HMWK*‡	3.1	22
Platelet lysate alone‡	<0.1	<0.1
Buffer + HMWK‡	2.5	2.9

*Supernatants from lysed platelets per milliliter (6.31 × 10^9) were used to make an 85% mixture with buffer (0.1 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4) and/or purified HMWK [stock 30 U/mL (20 μmol/L) or 12.7 U/mg]. Purified HMWK was diluted 1:10 in the reaction mixture. Samples were incubated for two hours at 37 °C.

†Leupeptin-treated lysate was prepared by the addition of leupeptin (0.5 mmol/L) ten minutes prior to the addition of purified HMWK.

‡Samples for HMWK coagulant assay were diluted 1:10 prior to testing. Data presented represent the mean results of three experiments.

Time course of activation of HMWK by a platelet protease. The activation of exogenous purified HMWK by platelet cytosol was studied in kinetic experiments. A 100,000 g cytosol of lysed platelets (8 × 10^9 platelets per milliliter) containing 1 mmol/L of EDTA was prepared in the presence or absence of leupeptin (Fig 7). After the addition of calcium (2.5 mmol/L), the coagulant activity of exogenous purified HMWK (final concentration of 2 U/mL or 12.5 U/mg before the addition of calcium) in the untreated platelet cytosol rose rapidly to 70 U/mL or 437 U/mg within ten minutes. This result indicated a 35-fold increase in coagulant activity. At longer incubations, the activity decreased, but at two hours the coagulant activity was 20 U/mL, ten times the original activity. No significant increase (2 to 4 U/mL) in the coagulant activity of exogenous HMWK added to the leupeptin-treated platelet cytosol was noted during the six-hour incubation.

Additional studies were performed to determine if bradykinin was released simultaneously with the cleavage of ^{125}I-HMWK and the increase in exogenous HMWK coagu-

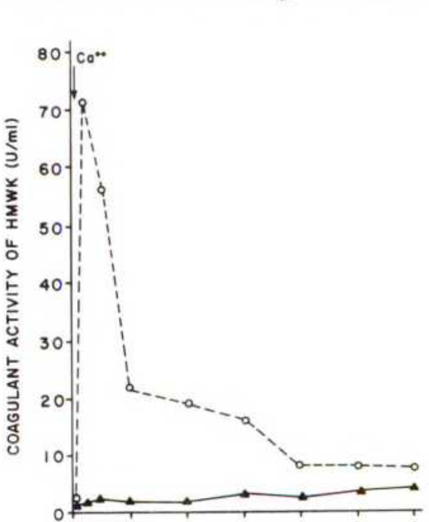

Fig 7. Time course of activation of HMWK coagulant activity by a platelet protease. A 100,000 g cytosol of platelets from 8 × 10^9 platelets per milliliter containing EDTA (1 mmol/L) was pretreated with leupeptin (0.5 mmol/L) (▲—▲) or left untreated (○---○). After the addition of purified HMWK [final concentration 2 U/mL (1.33 μmol/L)] into a mixture which consisted of 90% platelet cytosol in (0.1 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4) (TBS), the activation of HMWK was initiated by the addition of $CaCl_2$ (2.5 mmol/L). Samples were removed at 0 minutes, 10 minutes, 30 minutes, and at hourly intervals to determine the coagulant activity of HMWK. All incubations were performed at 37 °C.

lant activity. Under conditions similar to the coagulant activity studies of exogenous HMWK added to platelet cytosol, no bradykinin release was measurable. Because the possible amount of bradykinin released from the added HMWK could have been as much as 100-fold greater than the sensitivity of the radioimmunoassay to measure bradykinin, additional studies were performed to determine whether platelet cytosol contained an enzyme(s) that degraded bradykinin. It was found that platelet cytosol contained a DFP-sensitive enzyme that degraded exogenous bradykinin. Less than 10% of added bradykinin could be recovered from platelet cytosol, indicating that any released bradykinin by a calpain cleavage of HMWK in platelet cytosol would be rapidly proteolyzed.

DISCUSSION

The studies presented here indicate two potential pathways, perhaps interrelated, by which the interaction of HMWK with platelets may assume significance in the proteolytic reactions involved in contact phase activation. Platelet HMWK is expressed on the activated platelet surface and a platelet calcium-dependent cysteine protease(s) [Calpain(s)] is responsible for cleavage and increased coagulant activity of the HMWK molecule. Because platelet HMWK has been shown to be secreted from metabolically active platelets,[5] it was not surprising that the largest amount of the protein was found in the granule fractions enriched with α-granules in the unstimulated platelet (Figs 1 and 2). By radiolabeled HMWK tracer studies, the amount of HMWK found in the F_2 granule fraction must have been derived from the platelet granules themselves, since only 0.3% of a soluble HMWK tracer was attached to or trapped in the granule fraction. Furthermore, the amount of HMWK localized to the C/D α granule fraction must also have been derived from the platelet granules, since only 0.072% of the total soluble [^{125}I]HMWK was found to be associated with this fraction. The subcellular fractionation studies were confirmed by a study on the total amount of platelet HMWK in platelets from a patient with the gray platelet syndrome.[7] These platelets contained 16 ng/10^8 platelet HMWK, which is 28% of normal.

The finding that platelet HMWK is expressed on the external membrane of activated platelets (Fig 4) suggests one mechanism whereby a platelet form of a protein, which exists in the platelet in smaller quantities than in plasma, could be important. The expression of the platelet form of the protein on the activated platelet surface may be the means by which higher local concentrations of the protein may be achieved on the platelet surface than in plasma.[29] The validity of the modified CELISA as a quantitative indirect antibody consumption assay used in this study to determine the amount of membrane-expressed platelet HMWK is shown by comparison of the present results with our previously reported data.[5] In our previous study, we directly determined the amount of HMWK in supernatants of washed platelets to contain 2.8 ng HMWK/10^8 platelets.[5] In the present study, using the curve in Fig 3, we calculated that the supernatants of washed unstimulated platelets contain 4.2 ng HMWK/10^8 platelets. In addition, in the previous study, we estimated that washed platelets themselves have about 5 ng HMWK/10^8 platelets associated with the platelet suspension. In the present experiments, we estimate that washed platelet suspensions contain 4.9 ng HMWK/10^8 platelets. The similarity between these quantitative studies indicate that assessments as to the amount of platelet HMWK expressed on the activated platelet surface are valid.

In the present experiments, it was estimated that an amount of the total platelet HMWK equal to that which was secreted also was expressed on the activated platelet surface (Fig 4). This assessment is derived from the finding that activated platelets and their supernatant adsorbed similar amounts of the antibody to HMWK, as indicated by the similar decrease in the slopes of the competitive inhibition curves in Fig 4. Comparing the calculated slopes of the competitive inhibition curves produced by activated platelets and their supernatants in four experiments with the slope of the competitive curves produced by the antisera adsorbed with variable concentrations of purified HMWK (Fig 3), the amount of platelet HMWK expressed on the platelet surface and simultaneously secreted from platelets was ~17 ng/10^8 platelets. Comparing this value with the normal value of total platelet HMWK (60 ng/10^8 platelets),[5] ~28% of the total amount of platelet HMWK becomes secreted and independently expressed on the membrane of the activated platelet. This value for the amount of secreted platelet HMWK is lower than that (63%) which we previously reported for thrombin-activated platelets.[5] It is possible that the difference resulted from the threefold greater concentration of thrombin used for induction of the secretion reaction in the latter experiments. The presence of HMWK on the platelet surface suggests that the platelet, through the expression of its HMWK, may be an in vivo negatively charged surface for the regulation of the contact proteolytic system. Further studies are needed to determine if membrane-expressed platelet HMWK exists there in a high local density.

The finding that a platelet protease(s) cleaves HMWK (Figs 5 and 6) is not surprising, since calcium-requiring platelet proteases have been demonstrated to cleave other hemostatic cofactors: factor V, platelet fibrinogen, and von Willebrand factor.[30-32] In these studies with HMWK, a 100,000 mol wt cleavage product of HMWK along with lower mol wt bands are recognized (Figs 5 and 6). Because the cleavage of ^{125}I-HMWK consistently resulted in a 100,000 mol wt band and was inhibited by agents known to inhibit platelet calpain(s), but was unaffected by several other serine protease inhibitors that do not inhibit platelet calpain (Fig 6), it is likely that the cleavage of ^{125}I-HMWK observed in this study is a direct effect of calpain(s) and is not mediated through a secondary protease that is activated by calpain. Previous studies with purified proteins and in plasma indicated that kallikrein cleaves HMWK into heavy (64,000 mol wt) and light (46,000 mol wt) chains with an intermediate (56,000 mol wt) light chain.[4,33] Similar cleavage of HMWK occurs with factor XIa, although the initial cleavage of HMWK results in a 74,000 mol wt band.[34] The finding that a platelet cysteine protease(s) (Fig 6) is responsible for an additional 100,000 mol wt cleavage product of

HMWK would explain why this cleavage intermediate has not been recognized with purified plasma enzymes or in plasma when activated by a surface. The extent of proteolysis of ^{125}I-HMWK seen in these studies was probably dependent upon the amount of platelets that was used to prepare the cytosol. When there was a high concentration of platelet enzymes (Fig 5), both the 120,000 mol wt band and the 100,000 mol wt band of ^{125}I-HMWK were completely proteolyzed.

The finding that platelet calpain(s) can increase the specific coagulant activity of HMWK (Table 1, Fig 7) suggests that HMWK may be an important substrate for platelet calpains. The mechanism whereby platelet calpains increase the coagulant activity of HMWK is presently unknown. Because multiple bands are formed by platelet calpain cleavage of ^{125}I-HMWK, it is not clear which cleavage product is responsible for the measured increased specific coagulant activity of HMWK. In a previous study,[4] our group showed that kallikrein-cleaved HMWK had increased surface binding activity, and the amount of HMWK that adsorbed to an artifical surface was directly related to the expression of HMWK coagulant activity. That study suggests that proteolysis of HMWK, which exists as a procofactor, allows for increased surface-mediated activity. In that previous study,[4] it was not possible to make a direct measurement of the specific coagulant activity of the kallikrein-cleaved HMWK such as was performed in the present investigation since prekallikrein is in the substrate plasma of the coagulant assay and when activated on a surface must produce similar cleavages during the one-stage assay procedure. However, both studies suggest that proteolysis of the HMWK can augment its activity. The present investigation supports the hypothesis that HMWK exists as a procofactor. Additional studies with purified platelet calpain and HMWK or its light chains are needed to determine whether the 100,000 mol wt band or some other cleavage product appearing at the same time is responsible for the increased HMWK coagulant activity. Furthermore, since platelet cytosols contain an enzyme(s) that degrades bradykinin, neither was it possible to determine whether platelet calpain(s) releases bradykinin. Further studies are needed with purified platelet calcium-activated cysteine protease(s) on purified intact HMWK to confirm whether this enzyme(s) releases bradykinin.

The interaction between HMWK and platelet calpains may be more than fortuitous. The recognition that HMWK is a substrate for platelet calpain[35] has been corroborated with recent studies. Evidence by Ohkubo et al[36] and Muller-Esterl et al[37] indicate that α-2-thiol proteinase inhibitor—a major plasma proteinase inhibitor of cysteine proteases[38]—is identical to plasma low mol wt kininogen. Because the synthesis of the plasma kininogens is controlled by one gene,[39] the heavy chain of HMWK is identical to the heavy chain of low mol wt kininogen. HMWK, which differs from low mol wt kininogen by the presence of a light chain that contains its coagulant activity,[39,40] may be both a substrate and inhibitor of platelet calpains. Because it is well recognized that protease inhibitors are also substrates,[41] further studies are required to determine if purified HMWK inhibits purified platelet calpain.

Several laboratories have reported the presence of a calcium-dependent platelet protease(s)[42-46] which cleaves platelet membrane proteins,[43-46] alters endothelial prostaglandin production,[42] cleaves factor V,[30] fibrinogen,[31] and von Willebrand factor.[32] At least two calcium-dependent cysteine proteases are ubiquitously distributed in tissues, Calpain I and Calpain II, which differ in their requirement for activation by calcium; Calpain I requires μmol/L concentrations of calcium and Calpain II requires mmol/L concentrations.[47-49] To date, these proteases have been purified only from the cytosol fraction of the tissue from which it is derived.[42,48,49] How these calpains become available to alter their substrates, which are either on the platelet surface or contained within platelet granules, is unknown. However, these substrates may become altered by platelet calpains when platelets are disrupted by mechanical injury or complement-mediated cytolysis. This later notion would be consistent with our current hypothesis on the importance of HMWK. Because a deficiency of HMWK is not associated with any specific disease state, but the protein becomes altered to participate in the consequences of many disease states,[50] HMWK should be considered as a pathophysiologic mediator. HMWK in plasma or platelets could serve to inhibit platelet calpain(s) directly by its heavy chain and to neutralize the consequences of calpain's action indirectly by the formation of an activated cofactor form. In conclusion, these studies indicate that activated platelets express platelet HMWK on their surface that may be available to promote contact phase proteolysis on or at the platelet surface, and that platelets themselves contain a calpain protease(s) that can cleave HMWK and increase its coagulant activity and that might accelerate contact phase activation.

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Platelet-Derived Growth Factor Does Not Stimulate Prostacyclin Synthesis by Cultured Endothelial Cells

By Karleen S. Callahan, Anna Schorer, and John M. Harlan

We examined the effect of highly purified platelet-derived growth factor (PDGF) on prostacyclin (PGI_2) release by cultured human umbilical vein and bovine aortic endothelial cells. PDGF tested at concentrations equal to or exceeding those observed in serum did not increase endothelial cell PGI_2 synthesis as measured by radioimmunoassay of its metabolite, 6-keto-$PGF_{1\alpha}$. In contrast, cells incubated with 20% human whole blood serum (WBS) demonstrated significantly increased PGI_2 production (fivefold stimulation). Addition of anti-PDGF antibody to the 20% WBS did not attenuate the increased synthesis of PGI_2. Incubation with 20% plasma-derived serum (PDS) that was deficient in PDGF produced stimulation of PGI_2 release similar to 20% WBS. These results demonstrate that PDGF does not cause increased PGI_2 synthesis in cultured human endothelial cells of human or bovine origin, and further suggest that the stimulation observed with serum is not due to a platelet-release product.

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THE INTERACTION of platelets and endothelium with respect to thromboxane A_2 and prostacyclin (PGI_2) synthesis by these respective cell types has been extensively investigated. Although platelet-derived endoperoxides may supply substrate for endothelial prostaglandin formation,[1] the effects of platelet-derived proteins on endothelial cell prostanoid synthesis are not well defined.[2-6] Platelet activation by various physiologic stimuli results in the release of platelet α granule constituents including purified platelet-derived growth factor (PDGF), a potent mitogen which may contribute to wound repair following injury.[7-9] PGI_2, a major arachidonic acid metabolite produced by endothelium, is a potent vasodilator and inhibitor of platelet aggregation and release.[10] Previous investigations have reported increased prostaglandin synthesis in cultured cell systems including bovine aortic endothelial cells following incubation with PDGF,[11,12] and it was postulated that increased vascular PGI_2 production due to PDGF might act as a negative feedback mechanism for limiting platelet aggregation at sites of vascular injury.[4] The current study was designed to investigate this hypothesis by examining the effects of highly purified human PDGF,[13] as well as specific PDGF antibody, on PGI_2 synthesis in cultured human umbilical vein endothelial cells.

MATERIALS AND METHODS

Cell culture. Human umbilical vein and bovine aortic endothelial cells were isolated by collagenase treatment of vessels as previously described.[14] The human endothelial cells were maintained in 20% fetal calf serum (FCS), and the bovine cells were maintained in 10% FCS (Hyclone Laboratory, Logan, Utah) in either RPMI 1640 (human) or Waymouth's (bovine) medium (Grand Island Biologic Company, Grand Island, NY). Endothelial cells were passaged following trypsinization and plated at confluent density in 16-mm diameter wells (Costar, Cambridge, Mass). The human umbilical vein endothelial cells were used in first passage, and the bovine aortic endothelial cells were used in eighth passage. Bovine aortic smooth muscle cells at fifth passage were provided by Dr Stephen Schwartz, University of Washington, Seattle. Human foreskin fibroblasts were prepared as previously described[15] and were generously provided by Dr Russell Ross, University of Washington, Seattle.

Radioimmunoassay of endothelial cell 6-keto $PGF_{1\alpha}$ release. Prior to treatment, the monolayers were gently washed twice with 2.0 mL of serumless Neuman-Tytell medium which contains 0.2% lactalbumin (Grand Island). The wash medium was decanted and replaced with 0.5 mL of Neuman-Tytell medium containing control or test agents for an overnight incubation (24 hours) at 37 °C in 5% CO_2, 95% air. At the end of 24 hours, an aliquot of cell-free supernatant medium was removed from each well, placed into a polypropylene tube, and immediately frozen in a methanol-dry ice bath for storage at −20 °C until assayed. In selected experiments, the endothelial monolayers were gently washed again, the supernatant medium was decanted, and 0.5 mL of serumless Neuman-Tytell medium with or without purified human α-thrombin or A23187 was added for an additional 30-minute incubation. At the end of this time, the cell-free supernatant medium was removed and treated as above for later radioimmunoassay. Cell counts were performed on replicate wells by electronic particle counter (Particle Data, Inc, Elmhurst, Ill).

Measurement of unextracted, supernatant medium from the endothelial cell cultures for PGI_2 production was determined by radioimmunoassay (RIA) of the stable PGI_2 metabolite using [^{3}H]6-keto-$PGF_{1\alpha}$ (Amersham, Arlington, Ill), authentic 6-keto-$PGF_{1\alpha}$ standard kindly provided by Douglas McCarter and Dr John Pike, The Upjohn Company, Kalamazoo, Mich, and antiserum generously supplied by Dr William B. Campbell, University of Texas Health Science Center at Dallas. This anti-6-keto-$PGF_{1\alpha}$ antiserum cross-reacted <0.9% with PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGD_2, PGA_2, and TxB_2, 3.5% with 6, 15-diketo $PGF_{1\alpha}$, and 70% with 6-keto-PGE_1. The level of sensitivity was 10 pg/300 μL, and assays were run in duplicate and at more than one dilution when necessary. The proteins in the Neuman-Tytell medium did not interfere in the RIA and recovery of exogenous 6-keto-$PGF_{1\alpha}$ added to Neuman-Tytell medium was 106.7% ± 5.8%.

Preparation of whole blood serum (WBS) and plasma-derived serum (PDS). Human WBS was prepared following venipuncture from a single normal donor by allowing the blood to sit at room temperature until clotted (~two hours). The blood was then centrifuged at 2000 g for 20 minutes, and the cell-free serum was collected and passed through a 0.2 μm filter (Millipore, Bedford, Mass) in order to obtain a sterile preparation. The WBS was stored at 4 °C until used. PDS was prepared by collecting normal human blood from several donors into precooled syringes containing 3.5% citrate at a final dilution of 1:10 (vol/vol). The sample was immediately

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centrifuged at 2500 g for 20 minutes at 4 °C. The cell-free and platelet-free plasma was recalcified, dialyzed against phosphate-buffered saline (PBS) to remove citrate, centrifuged to remove the clot, and stored at 4 °C.

Radioreceptor assay of PDGF. Levels of PDGF in WBS and PDS were determined by radioreceptor assay using human foreskin fibroblasts and ^{125}I-labeled PDGF as described by Bowen-Pope and Ross.[15] In brief, confluent fibroblast monolayers were put on ice and rinsed with cold PBS containing 0.1% bovine serum albumin (BSA) and 0.5 mmol/L of CaCl, pH 7.4. This binding rinse was aspirated from the cells and replaced with 1.0 mL per well of test solution for a three-hour incubation at 4 °C with constant shaking. Next, the supernatant was removed and replaced with 1.0 mL per well of 25 mmol/L of Hepes buffer, pH 7.4, containing 0.25% BSA and 0.5 ng ^{125}I-PDGF. The fibroblasts were incubated at 4 °C with shaking for one hour, the supernatant was aspirated, and the cells were washed twice with the binding rinse to remove unincorporated ^{125}I-PDGF. Finally, the cells were solubilized with 0.1% Triton X-100 (New England Nuclear, Boston) in water containing 0.1% BSA and were collected for gamma counting. This radioreceptor assay detects levels of >0.1 ng PDGF/1.0 mL.

Reagents. Highly-purified PDGF[13] and goat anti-human PDGF antibody were generously supplied by Dr Elaine Raines and Dr Russell Ross, University of Washington, Seattle. The PDGF preparation was a 500,000-fold purification from serum and consists of four components with mol wts of 27,000 to 31,000 in the nonreduced state using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). PDGF was stored in 10 mmol/L of acetic acid containing 0.25% BSA, which was diluted a 100-fold or more in Neuman-Tytell media for experiments. A comparable volume of this acetic acid solution was added to control cultures. The IgG fraction of goat anti-human PDGF antiserum was prepared by 18% Na_2SO_4 precipitation and diethylaminoethanol (DEAE)-sephacel chromatography. The anti-PDGF IgG (at a concentration of 20 μg/mL) completely inhibits 1 ng/mL purified PDGF or an equivalent amount of PDGF in WBS as determined by mitogenic activity or ability to compete with ^{125}I-PDGF in the radioreceptor assay (E. R., personal communication). Purified human α-thrombin (12,000 U/mg protein) was generously provided by Dr John Fenton II, (Buffalo). A23187 was obtained from Sigma Chemical Company.

Statistical analysis. The results were evaluated by analysis of variance with the Neuman-Keul's multiple comparisons test.

RESULTS

Figure 1 shows the effects of WBS and PDS on endothelial PGI_2 synthesis as measured by RIA of 6-keto-$PGF_{1\alpha}$. Incubation of the human endothelial cell monolayers with 20% human WBS resulted in a significant increase in 6-keto-$PGF_{1\alpha}$ release when compared with incubation in serumless medium alone. Comparable stimulation was observed with WBS prepared from a pool of donors. Addition of the anti-PDGF IgG to 20% WBS did not attenuate the stimulatory effect. Anti-PDGF IgG alone did not increase 6-keto-$PGF_{1\alpha}$ synthesis over that observed with medium alone (data not shown). Addition of 20% PDS, however, caused a significant increase over medium alone (Fig 1). There was no further stimulation over that observed with PDS alone when PDGF was added to 20% PDS (data not shown). The concentration of PDGF in WBS was 17.0 ng/mL, 1.3 ng/mL in WBS containing anti-PDGF IgG, and <0.1 ng/mL in PDS.

In contrast to the results obtained with WBS or PDS, a 24-hour incubation of human endothelial cells with purified PDGF (10 to 100 ng/mL) did not result in stimulation of 6-keto-$PGF_{1\alpha}$ production (Fig 2). The reported values of PDGF in human whole blood serum are 17.5 ng/mL by radioreceptor assay[16] or 50.0 ng/mL by radioimmunoassay.[17]

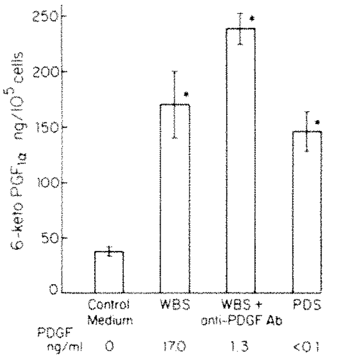

Fig 1. Effect of whole blood serum (WBS) and plasma-derived serum (PDS) on 6-keto-$PGF_{1\alpha}$ release. Human umbilical vein endothelial cell monolayers were incubated with medium alone (n = 16), medium containing 20% WBS (n = 12), 20% WBS with anti-platelet-derived growth factor (PDGF) antibody (Ab) (n = 8), or 20% PDS (n = 8). Aliquots of supernatant medium were removed after 24-hour incubation for determination of 6-keto-$PGF_{1\alpha}$ levels by RIA. Values represent means ± 1 SE from quadruplicate wells in two to four individual experiments (* $P < .01$). The levels of PDGF in the serum preparations were determined by radioreceptor assay.

We also examined the effects of platelet products on subsequent thrombin-induced PGI_2 synthesis as shown in Fig 3. The cells were incubated for 24 hours with various treatments, washed once, and incubated with fresh medium with or without α-thrombin for 30 minutes. α-Thrombin induced a marked increase in 6-keto-$PGF_{1\alpha}$ synthesis in cells preincubated in medium alone and in cells preincubated with either PDGF or WBS. In contrast, monolayers previously incubated with α-thrombin were refractory to subsequent stimulation by α-thrombin, as reported by others.[18]

Because the previous report of PDGF-stimulated endothelial cell PGI_2 release used bovine aortic endothelial cells,[4] we

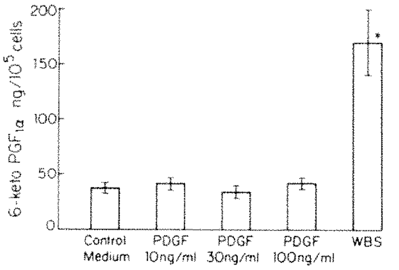

Fig 2. Effect of platelet-derived growth factor (PDGF) on 6-keto-$PGF_{1\alpha}$ release. Human umbilical vein endothelial cell monolayers were incubated with medium alone (n = 16), medium containing PDGF 10 ng/mL (n = 16), PDGF 30 ng/mL (n = 8), PDGF 100 ng/mL (n = 4), or medium containing 20% WBS (n = 8). Aliquots of supernatant medium were removed after 24-hour incubation for determination of 6-keto-$PGF_{1\alpha}$ levels by RIA. Values represent means ± 1 SE from quadruplicate wells in one to four experiments (* $P < .01$).

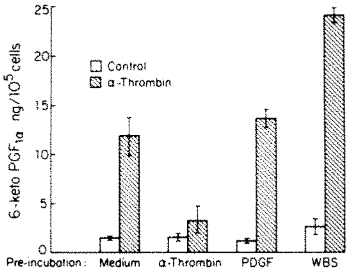

Fig 3. Effect of platelet-derived proteins on thrombin-stimulated 6-keto-PGF$_{1\alpha}$ release. Human umbilical vein endothelial monolayers were incubated for 24 hours with medium alone, medium containing 8 U/mL α-thrombin, 10 ng/mL PDGF, or 20% WBS. The monolayers were then washed and incubated with medium alone or medium containing α-thrombin (8 U/mL). Aliquots of supernatant medium were removed after 30-minute incubation for determination of 6-keto-PGF$_{1\alpha}$ levels by radioimmunoassay. Values represent means ± 1 SE from quadruplicate wells in two experiments.

repeated similar experiments using our PDGF preparation and bovine aortic endothelial cells (Table 1). Although PGI$_2$ synthesis in these multiple passages of bovine endothelial cells was considerably less than in the human endothelial cells, addition of PDGF (10 to 100 ng/mL) did not increase 6-keto-PDF$_{1\alpha}$ release when compared with serumless medium alone. These same bovine cells were able to respond to subsequent stimulation using calcium ionophore A23187 or 20% WBS (data not shown).

Finally, we examined the effect of PDGF on PGI$_2$ synthesis in another vessel wall cell type. In contrast to human or bovine endothelial cells, bovine aortic smooth muscle cells demonstrated increased 6-keto-PGF$_{1\alpha}$ synthesis following incubation with PDGF (Table 1). PDGF caused a greater than tenfold increase in 6-keto-PGF$_{1\alpha}$ release in smooth muscle cells. In addition, 6-keto-PGF$_{1\alpha}$ synthesis was significantly increased in smooth muscle cells exposed to 20% WBS when compared with those incubated in 20% PDS. Although addition of anti-PDGF IgG to WBS resulted in decreased PGI$_2$ synthesis as compared with WBS alone, the amount released was still significantly higher than with medium alone (Table 1).

Table 1. Effect of PDGF on Bovine Vessel Wall Cell PGI$_2$ Release

		6-keto-PGF$_{1\alpha}$ ng/10^5 Cells*
Addition to bovine aortic endothelial cells		
Control	(3)	1.34 ± 0.22
PDGF		
10 ng/mL	(3)	0.80 ± 0.06
50 ng/mL	(3)	0.96 ± 0.06
100 ng/mL	(3)	0.99 ± 0.13
Addition to bovine aortic smooth muscle cells		
Control	(4)	0.32 ± 0.04
PDGF		
10 ng/mL	(4)	4.02 ± 0.14*
20% WBS	(4)	18.84 ± 0.68*
20% PDS	(4)	5.14 ± 0.50*
20% WBS + anti-PDGF Ab	(4)	14.24 ± 0.88*

PDGF, platelet-derived growth factor; WBS, whole blood serum, PDS, plasma-derived serum.

6-Keto-PGF$_{1\alpha}$ levels in supernatant medium were determined by RIA after a 24-hour incubation. Values represent means ± 1 SE of (n) replicate wells.

*$P < .01$.

DISCUSSION

Previous investigations have demonstrated that PGI$_2$ production by endothelial cells is stimulated by unidentified factor(s) present in normal plasma and serum.[19-23] Coughlin et al,[4] using cultured bovine aortic endothelial cells, demonstrated that serum prepared from platelet-rich plasma produced a greater increase in PGI$_2$ release than did serum prepared from platelet-poor plasma. The serum factor described in that report was similar to PDGF in being heat-stable and nondialyzable; they also found that addition of partially purified PDGF resulted in significant enhancement of PGI$_2$ synthesis within 24 hours. These investigators suggested that PDGF or other products released by activated platelets might provide feedback inhibition of further platelet aggregation by stimulating endothelial PGI$_2$ production.[5]

We did not observe an increase in PGI$_2$ production when cultured human umbilical vein or bovine aortic endothelial cells were incubated with purified PDGF at concentrations equal to or exceeding normal serum levels (Fig 2 and Table 1). Although incubation with WBS significantly increased PGI$_2$ synthesis, PDS that lacked PDGF produced a similar increase. Furthermore, addition of specific anti-PDGF IgG to the WBS did not attenuate the enhanced PGI$_2$ generation (Fig 1). These observations demonstrate that PDGF is not the stimulating factor in serum responsible for enhanced PGI$_2$ synthesis by cultured endothelial cells.

We also found that prior incubation of the endothelial cells with either PDGF or WBS did not attenuate subsequent stimulation of PGI$_2$ induced by α-thrombin (Fig 3). These observations suggest that platelet-derived products do not down regulate endothelial PGI$_2$ synthesis as has been reported for β-thromboglobulin.[2] PGI$_2$ synthesis by bovine aortic smooth muscle cells was stimulated by purified PDGF, and 20% WBS resulted in greater production of PGI$_2$ than did 20% PDS. In addition, PGI$_2$ production was reduced by the addition of anti-PDGF IgG to WBS. Thus, as previously reported by Coughlin et al,[5] smooth muscle cell PGI$_2$ synthesis is sensitive to platelet-derived products, including PDGF. In all of these studies, it should be noted that WBS and PDS differ in more than platelet release products. Products of leukocytes activated during clotting ex vivo could contribute to the PGI$_2$ stimulatory activity of WBS.

Although our results on endothelial cell response to PDGF differ from those of Coughlin et al,[4] they are similar to those of Poggi and co-workers.[6] These latter investigators examined cultured bovine aortic endothelial cells and reported that partially purified PDGF had an insignificant effect on PGI$_2$ synthesis at 24 hours (140.7% of control). Both of these earlier investigations[4,6] utilized partially purified PDGF prepared according to the method of Antoniades et al,[24] whereas the present study used more highly purified PDGF prepared according to the method of Raines and Ross.[12] Consequently, discrepancies among the studies may be due in part to differences in the PDGF preparations and the possibility of a contaminating serum factor in the earlier studies. Our preparation of PDGF was active as a mitogenic factor in fibroblasts and did stimulate PGI_2 production by cultured smooth muscle cells.

The fact that cultured human umbilical vein and bovine aortic endothelium lack PDGF receptors[25,26] would argue against PDGF-mediated effects on PGI_2 release from these cells although it is possible that PDGF could act by receptor-independent mechanisms. For example, although only native PDGF binds to its receptor and stimulates mitogenesis,[27] both native and reduced PDGF stimulate leukocyte chemotaxis.[28] This observation suggests that receptor binding may not be essential for all cell responses to PDGF.

In conclusion, purified PDGF did not stimulate PGI_2 production in cultured endothelial cells of either human or bovine origin, and an anti-PDGF antiserum did not inhibit PGI_2 generation induced by WBS. Plasma-derived serum with an undetectable level of PDGF also caused significant enhancement of endothelial PGI_2 synthesis, providing further evidence that PDGF is not responsible for the stimulatory effect of human serum on endothelial cell PGI_2 synthesis.

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Clinical and Biologic Features Predict A Poor Prognosis in Acute Lymphoid Leukemias in Infants: A Pediatric Oncology Group Study

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Analysis of remission induction rates for 1,117 children 18 months to 10 years of age (group 1) and 90 infants <18 months of age (group 2) with acute lymphoid leukemia (ALL) and of duration of continuous complete remission (CCR) for 454 in group 1 and 33 in group 2 revealed that infants fared significantly worse in both measures of outcome ($P = .03$ and $P < .0001$). To examine potential reasons for the poor prognosis of affected infants, clinical and biologic features of their ALL were compared. Infants had higher WBC counts ($P < .001$), a higher incidence of massive splenomegaly ($P < .001$), massive hepatomegaly ($P < .001$), more central nervous system (CNS) disease at diagnosis ($P < .01$), and lower platelet counts ($P < .001$). Also, their blasts were less often PAS$^+$ ($P = .02$). The incidence of non(T, B, pre-B), T and pre-B immunophenotypes of ALL did not differ significantly between the two groups. However, in patients with non(T, B, pre-B) ALL, the majority (51%) of infants had common ALL antigen (CALLA)-negative blasts, as compared with only 7% in group 1 ($P < .001$). Furthermore, infants with non(T, B, pre-B) cell ALL who were <12 months of age were almost always CALLA$^-$ (18 of 21). The blasts of children from both groups usually expressed Ia-like antigens. These data illustrate that infants with ALL have extensive and bulky disease more often than do older children and are more often affected with a prognostically unfavorable phenotype of acute leukemia (AL) which expresses Ia-like antigens but is more often PAS$^-$ and CALLA$^-$. We believe that these clinical and biological differences predict and explain in part the observed poor response to treatment of infants with ALL.

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THE PROGNOSIS of children with acute lymphoid leukemia (ALL) has improved remarkably during the last decade. Presently, >50% of newly diagnosed children may be expected to survive. However, many children continue to fail treatment and eventually succumb to their disease. The ability to predict which children with ALL have a high probability of treatment failure has recently become possible through the definition of certain prognostic factors that may be determined at the time of diagnosis. These include such clinical or biologic factors as the level of the white blood cell (WBC) count,[1-3] age,[3] presence of organomegaly,[1,2,4-6] gender,[1,2,5,7] tumor cell ploidy and/or karyotype,[8,9] platelet count,[1,10] presence of a mediastinal mass,[2,4,11,12] race,[10,13,14] cell morphology,[15-19] immunophenotype,[3,12,20-24] rapidity of achieving bone marrow remission,[15] the presence of central nervous system (CNS) leukemia at the time of diagnosis,[2,4] glucocorticoid receptor levels in blasts,[25] and blast PAS positivity.[26] Furthermore, the relative importance of many of these prognostic factors has been shown to be dependent on the immunophenotype of ALL[3] and the specific therapy given.[27] This ability to predict which children have a high probability of treatment failure, made possible by prognostic factor analysis, has permitted more individualization of therapy.

One of the most powerful predictors of prognosis in most studies is the age of the affected patient. Patients >10 or 11 years of age or <1 to 2 years of age have been noted to fare much worse than children who are between 1½ and 11 years old at the time of diagnosis.[3,28] The reasons for treatment failure in affected patients who are younger or older than average at the time of diagnosis are presently unknown. Some investigators have reported unusually severe drug-associated or radiation-associated toxicity in infants.[29,30] Often, a recommendation for a reduced drug dose is made in these young patients. Nevertheless, it is unknown whether or not such problems contribute to the high relapse rate of affected infants; a recent report suggests that drug dose reduction is not the explanation.[30]

To examine the hypothesis that infants in our studies might have fared worse because of clinical and/or biologic differences in their leukemia, the Pediatric Oncology Group (POG) retrospectively examined their data regarding clinical and biologic features of children (<11 years of age) affected with ALL including: the levels of the WBC and platelet counts, the Hgb level, the presence of massive organomegaly, the incidence of CNS disease at diagnosis, the sex ratio, race, immunophenotype distribution, cytogenetic profiles, French-American-British (FAB) classification, expression of Ia-like antigen and common acute lymphocytic leukemia antigen (CALLA), glucocorticoid receptor levels in blasts, and periodic acid-Schiff (PAS) expression in blasts. We found that infants fared much worse than older children (group 1) with ALL who were given the same therapy and had evidence of more bulk disease, including CNS leukemia at diagnosis. They also often had a biologically different type of acute leukemia (AL) which was usually of L1 morphology and expressed Ia-like antigens, but

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was more often PAS$^-$ and CALLA$^-$ than ALL affecting older children.

MATERIALS AND METHODS

After informed consent was obtained, 1,566 newly diagnosed children <11 years of age with esterase-negative and Sudan black B-negative acute leukemia were evaluated between May 1979 and May 1984. The <18-month age cutoff was used to define group 2 because previous POG analyses of its children with ALL revealed a worse prognosis for children who were below this age at the time of diagnosis. Arbitrarily, adolescents (>10 years of age) were excluded from this analysis and will be the subject of a later report. In addition to the routine history, physical examination, laboratory, and radiographic studies which were performed on all patients ($N = 1566$), detailed classification studies were done on lymphoblasts from most children ($n = 936$) at the reference laboratories of the University of Alabama at Birmingham, Duke University Medical Center and John's Hopkins Steroid Receptor Laboratory on leukemia cells obtained at diagnosis from each patient.[31] Only 1,207 (1,117 in group 1 and 90 in group 2) children who were evaluable and who had uniform remission induction therapy with vincristine and prednisone ± L-asparaginase on POG 7623, 7866 or 8036 treatment protocols were used for the analysis of remission induction rates. The subset of 487 affected children (33 infants) with non-B, non-T cell ALL who had been followed for adequate periods of time (see Results section) and were treated on the POG 7623 (ALinC 12) study (see Appendix for details) were used for the analyses of durations of complete remissions.

Immunologic and cytochemical cell markers and glucocorticoid receptors. Wright's–Giemsa–stained leukemic cells were examined morphologically by a panel of five collaborating hematologists and one pathologist and were classified according to FAB criteria.[32] In addition, leukemic cells were examined for Sudan black B,[33] PAS, non-specific esterase, and chloroacetate esterase cytochemical reactivities by standard techniques, for surface immunoglobulin (SIg),[34] and for receptors for sheep erythrocytes (E-R),[35-37] IgG, and for the third component of complement[21,38] by established and quality-controlled techniques at member institutions.[31] At each member institution, cells were stained for SIg, centrifuged onto glass slides, fixed in cold 95% ethanol–5% acetic acid, washed, and mailed submerged in phosphate-buffered saline (PBS) to the University of Alabama at Birmingham's Immunology Reference Laboratory for cytoplasmic immunoglobulin (CIg) testing, as previously described.[39] Heparinized bone marrow samples were placed in tissue culture media and were shipped by express mail to Duke University's Immunology Reference Laboratory to be examined for the expression of Ia-like (HLA-DR) and pan-T (pT) cell markers and CALLA by microcytotoxicity assays using heteroantisera and monoclonal antibodies, as described.[40-42] Glucocorticoid levels in blast cells were also determined.[25]

Definitions. All leukemias were classified into T, B, pre-B or non(T, B, pre-B) cell ALL subgroups according to the following criteria: pre-B cell, if $\geq 10\%$ of marrow lymphoblasts contained CIg; B cell, if $\geq 10\%$ of marrow lymphoblasts had easily detected SIg without cIg (ie, μ heavy chains); T cell, if $\geq 40\%$ of marrow lymphoblasts were lysed by pan-T (pT) heteroantisera (40% above control lysis by cytotoxicity testing). The non(T, B, pre-B) cell group included all patients with ALL not classified by the above criteria, irrespective of the expression of CALLA and Ia-like antigens. However, most of these latter cases expressed Ia-like antigens (97%); overall, most expressed CALLA (90%).

Statistics. Although some inferences are phrased in the text as though one-sided comparisons were made, all significance levels (P-values) reported resulted from two-sided comparisons. The method of Kaplan and Meier was used to construct the life tables and curves.[43] The Mantel-Haenszel statistic was used to compare life tables.[44] If expected cell frequencies permitted, contingency tables were analyzed using the classical chi-square statistic.[45] Otherwise, an exact procedure, based on the chi-square statistic, was used.[46] Distribution of quantitative factors (such as platelet counts and WBC counts at diagnosis) were also analyzed using the Wilcoxon test.[45]

RESULTS

Response to treatment. Forty-seven of 1,117 (4%) children from group 1 and 9 of 90 (10%) infants from group 2 who were evaluable failed to achieve a complete remission in four weeks with vincristine and prednisone (VP) or VP and L-asparaginase therapy ($P = .03$). The duration of continuous complete remission (CCR) for the 454 evaluable children from group 1 and 33 children from group 2 who entered remission and were maintained on POG 7623 (AlinC 12) treatment regimens (described in Appendix) is shown in Fig 1. The median duration of follow-up for those children who remain at risk is three years and 11 months. The duration of CCR for those who were treated according to POG 8036 are not included here since the follow-up time for this study is

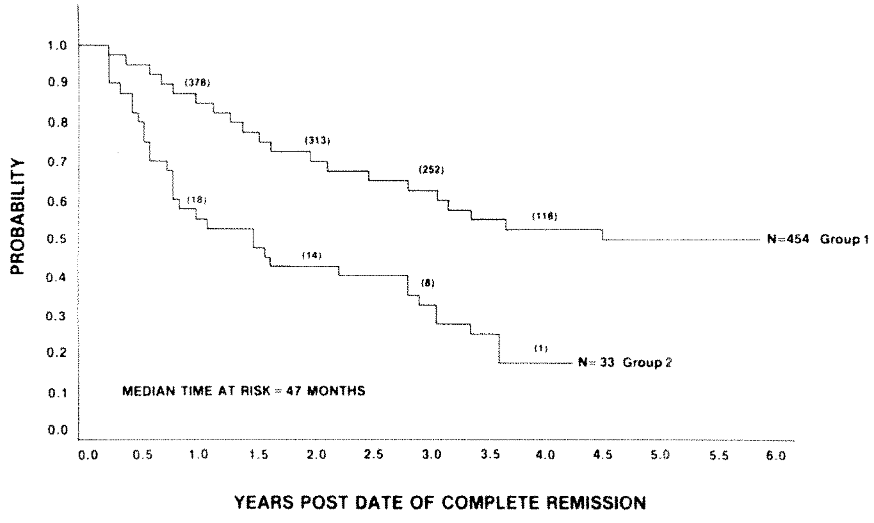

Fig 1. Duration of continuous complete remission (CCR) for patients treated according to POG 7623 with non-T, non-B, acute lymphoid leukemia ($P = .0001$). Failure is defined as relapse at any site or death while in remission. The number of patients who remain at risk at various points in time are noted in parentheses. Group 1, patients >10 years of age; group 2, patients <18 months of age.

short (median = 12 months). It may be seen that infants fare far poorer than do older children $P = .0001$. They also have significantly shorter durations of both bone marrow and CNS remissions ($P < .03$ and $P < .001$, respectively). The three-year CNS remission rate was 66% (SE = .11) for infants and 89% (SE = .02) for children in group 2 and the 3-year bone marrow remission rate for infants was 64% (SE = .11); for children in group 2, it was 74% (SE = .02).

Comparisons of possible clinical prognostic factors between age-defined subgroups of children with ALL. Data in Table 1 illustrate that the infant group is more likely to have a significantly larger proportion of affected individuals who have a high WBC count at diagnosis. Infants also have a higher incidence of massive (respective organ below the umbilicus) hepatomegaly (18% *v* 6%) and splenomegaly (23% *v* 8%) ($P < .001$ and $P < .001$, respectively), lower median platelet counts (36,000/μL *v* 52,000/μL) ($P < .001$) and more CNS disease at diagnosis (11% *v* 4%, $P = .003$). In contrast, there was no significant difference in their sex ratio, racial distribution, incidence of a mediastinal mass, hemoglobin levels at diagnosis, percentage of marrow blasts, or incidence of bone involvement as compared with older children. There was no significant difference in the proportion of infants (29 of 46, 63%) *v* older children (374 of 513, 73%) who were in complete remission at day 14 of induction treatment ($P = .08$) after treatment on POG 8036; however, the number of infants available for analysis was small. Data from patients treated according to POG 8036 were used for this latter analysis, since bone marrow examinations were required on day 14 of treatment on this study only.

Comparisons of laboratory-defined features (group 1 v *group 2).* Data in Table 2 illustrate that infants with non(T, B, pre-B) ALL are more often CALLA$^-$ than older children (group 1), but usually express Ia-like antigens (38 of 39). Furthermore, the data show that most of the children who were CALLA$^-$ (group 2) were in the group who were <1 year of age at diagnosis. In addition, infants (group 2) with pre-B cell ALL had blasts that were CALLA$^-$ significantly more often than those from similar cases of pre-B cell ALL in older children (see Table 2). Table 2 also illustrates that there were no significant differences in distributions within the lineage-specific immunologic groups shown between group 1 and group 2, and between group 1 and group 2 infants <1 year of age. The small group of 12 children with B cell ALL were excluded from this analysis. Table 3 summarizes the limited cytogenetic data available for both groups 1 and 2. No significant difference in ploidy distribution or other cytogenetic features were noted. However, there was a high incidence of unsatisfactory studies and of "normal" results. It is possible that poor-quality banding studies, which were frequently noted, obscured significant differences between the patient groups regarding specific cytogenetic abnormalities such as translocations. No significant differences in the levels of corticosteroid receptors in blast cells or L1 or L2 morphology of the blasts from these patients were observed between patient groups. The blasts from infants, however, were significantly more often PAS– ($P = .02$).

Table 1. White Blood Cell Count Distribution By Age Groups

WBC per μL	Group 1 No of Patients (%)	Group 2 Infants (%)
<10,000	705 (49)	32 (29)
10,000–50,00	482 (33)	27 (24)
50,000–100,000	132 (9)	27 (24)
≥100,000	135 (9)	26 (23)
$P < .0001$		POG 7865 and 8035

DISCUSSION

This study has demonstrated that infants have a much worse prognosis than do children who are older. They enter remission less readily (n = 1,207, $P = .03$) and survive in CCR for shorter periods of time than older children (n = 487, $P = .0001$) (see Fig 1). Infants also have a higher incidence of CNS disease at diagnosis and experience shorter durations of both CNS and bone marrow remissions ($P < .001$ and $P < .03$). Our data also illustrate that infants have more bulk disease (hepatosplenomegaly and higher WBC counts initially) and lower platelet counts than do children from group 1. All of these factors have been associated with a poorer prognosis in other studies.[1,2,4-6,10]

Immunologic marker analysis of the blasts from the infants (group 2) in our study revealed that approximately two-thirds of them have ALL of well-defined cell lineage based upon detection of specific cell markers (CIg$^-$, SIg$^-$, CALLA$^-$, or T cell-specific antigens). The distribution of these well-defined phenotypes of ALL is not significantly different from that seen in older affected children except for the lower incidence of CALLA expression in group 2. However, one third of these infants have AL of FAB L1 morphology, which expresses Ia-like (HLA-DR) antigens but lacks CALLA and CIg expression (see Table 2) and is more often PAS negative. Furthermore, if one considers only infants <1 year of age, more than one-half have CALLA$^-$, CIg$^-$, SIg$^-$, T-antigen$^-$, HLA-DR$^+$ acute leukemia. The lack of CALLA expression by blast cells has been associated with a poor prognosis in some studies.[21,28] No other similarly studied group of affected infants is available for comparison, and only occasional case reports of individual infants who were similarly studied are available.[47] Taken together, these clinical and biologic findings supports the notion that infants often have a biologically different type of acute "undifferentiated" leukemia which appears to be more aggressive than the disease seen in older children and is less responsive to the treatments commonly used for ALL.

Nadler et al[48] have examined B cell-associated and B cell-restricted antigens and, in some cases, the state of the immunoglobulin genes of tumor cells from 138 children and adults with non-T cell ALL. They found that leukemic cells from the patients could be assigned to one of four maturational groups based on sequential patterns of antigen expression: HLA-DR–related Ia-like antigen (Ia) alone (stage I); Ia/B4 (stage II); Ia/B4/CALLA (stage III); and Ia/B4/CALLA/B1 (stage IV). The expression of B cell-restricted antigens (B4 and B1) and rearrangements of Ig heavy chain

Table 2. Incidence of Immune Phenotypes of ALL Within Age Groups of Affected Children

Immunophenotype	Group 1 1.5 to 10 yr No. of Children (%)	Group 2 <1.5 yr No. of Children (%)	Group 2 Patients <1 yr No. of Children (%)
A. Non(T, B, Pre-B)	591 (67)	39 (65)	21 (64)
CALLA$^+$*	544 (93)	19 (49)	3 (14)
CALLA$^-$	40 (7)	20 (51)	18 (86)
B. Pre-B†	154 (18)	14 (23)	7 (21)
C. T	122 (14)	4 (7)	3 (9)
D. B	9 (1)	3 (5)	2 (6)
Total	876	60	33

Statistical analysis of data: comparison of immune phenotype distributions (A v B v C) between groups 1 and 2 ($P > .9$) and between group 1 and group 2 patients <1 year of age ($P < .7$]. (B cell patients excluded because of small numbers.) Comparison of distributions of CALLA expression for group 1 v Group 2: $P < .0001$ for children with non(T, B, pre-B) cell ALL; $P = .01$ for children with pre-B cell ALL (see data below).

Ninety-seven percent of patients in group 1 with non(T, B, pre-B) cell ALL expressed Ia-like (HLA-DR) antigens as compared with 97% (38 of 39) of infants (group 2).

*Distribution of CALLA expression by 6-month increments of age in children with non(T, B, pre-B) cell ALL <6 months: 9 CALLA$^-$, 1 CALLA$^+$: 6 to 12 months: 9 CALLA$^-$, 2 CALLA$^+$; 12 to 18 months: 2 CALLA$^-$, 16 CALLA$^+$.

†Ninety-three percent of pre-B cell leukemias expressed CALLA (Group 1, 96%, Group 2, 71%, group 2 infants <1 year of age, 57%).

genes provided strong evidence for the B cell lineage of stages II, III, and IV tumors. They also found normal counterparts for leukemic cells of stages II through IV among fetal and adult bone marrow cells. They concluded that the 4% of their patients who had blasts that expressed only Ia-like antigens (stage I) had leukemias of uncertain lineage, but they speculated that the blasts from this group represented an even earlier stage of pre-B cell differentiation than did those from the IaB4$^+$ subgroup (stage II). No blasts of the stage I patients expressed CIg and results of analyses for immunoglobulin gene rearrangements were not reported for that group. The blasts from 97% of our infants with non(T, B, pre-B) ALL expressed HLA-DR (Ia-like) antigens, but the leukemia cells of 51% of these infants with non(T, B, pre-B) cell ALL did not express CALLA. The blasts from these cases may be analogous to Nadler's stage I, but we cannot say how many are in each stage because testing with the monoclonal antibody B4 was not done. In addition, the blasts of several of our cases were HLA-DR$^+$, CIg$^+$, and CALLA$^-$. This phenotype does not follow the maturational sequence proposed by Nadler, since the expression of CIg would be expected to occur concomitant with or after the expression of CALLA. This may represent an aberrant phenotype. Further studies are needed to define more clearly the cell lineage and maturational stage of the leukemic cells of this group of patients with AL. Blasts from one of the affected infants with AL included in this study have been examined to determine the state of the Ig genes and expression of terminal deoxynucleotidyl transferase (TdT), the blasts were also examined by electron microscopy (EM). The Ig heavy chain genes were rearranged, TdT was expressed, and EM findings compatible with common or pre-B cell ALL were observed, suggesting that this case was of early B cell lineage.[49] Similar studies of the blasts from more of these infants would be of interest.

Table 3. Results of Cytogenetic Studies Within Age Groups of Children With ALL

Ploidy	Group 1 1.5-10 yr (%)	Group 2 <1.5 yr (%)
Normal	316 (63)	24 (59)
Hyperdiploid	107 (21)	11 (27)
Hypodiploid	25 (5)	1 (2)
Pseudodiploid	40 (8)	5 (12)
Constitutional abnormality*	14* (3)	0

Distribution overall: P = NS; pseudodiploidy, group 1 v group 2: $P = .37$.

*The 14 constitutional abnormalities were: 11 Downs' syndrome, 1 Klinefelter's syndrome, 2 others.

Reaman et al[30] have presented data regarding certain clinical features and the outcome of 115 infants (<1 year of age) treated on Children Cancer Study Group (CCSG) protocols. These infants had higher WBC counts and more CNS disease at diagnosis than did the older children in their studies. The infants with CNS disease fared poorly: 82% subsequently experienced a bone marrow relapse. Furthermore, although 96.5% of these infants achieved complete remission, only 30 of them did so by day 14 of induction, and the induction death rate was 4%. Drug dosage modification occurred in only 9% of these infants. The median duration of remission was 8.5 months. In addition, there was a similarly high CNS relapse rate in infants who received cranial irradiation (22%) or only intrathecal chemotherapy (23%) as a part of their CNS prophylaxis. The testicular relapse rate was 6%. Relapse-free survival at three years from diagnosis was only 12.5%, with only eight infants continuing in CCR >1 year following cessation of therapy. Severe neuropsychologic side effects were noted in at least 50% of these infants.

The findings in our population of infants with ALL are similar to those of Reaman et al in that the WBC counts of the patients (see Table 2) were higher, as was their incidence of CNS disease (11%) and CNS relapse rate (24%). The 8.5-month median duration of remission noted in the infants reported by Reaman et al is similar to the 10-month median duration of remission noted in our infants who are <1 year of age. The CCR rate at 3 years of the infants in the CCSG study was 12.5%, which may be compared to a CCR rate of 25% at 3 years and 8% at 4 years in our infants who were <1 year of age. We do not have specific data regarding neuropsychologic side effects and drug dosage received in our patients. The clinical findings of the infants in our study extend those reported by Reaman et al in that we noted a significantly higher incidence of massive hepatosplenomegaly and lower platelet counts.

It is clear from our studies that infants with morphological criteria, with or without immunologic criteria for the diagnosis of ALL, have a very poor prognosis when treated with chemotherapy designed for children with ALL (Fig 1). Our infant population entered remission less well and experienced more CNS and bone marrow relapses than did older children. The immunologic marker data revealed that approximately two thirds of these infants have AL of well-defined lymphoid lineage, whereas one third of them have an AL of uncertain lineage. The latter expresses Ia-like antigens without CALLA or CIg and is more often PAS$^-$ than is the ALL seen in older children. These infants appear to have a biologically different form of AL, the lineage of which remains uncertain at present. We believe that the biologic and clinical differences noted in these infants with ALL predict and explain in part their poor prognosis. Clearly, better therapy is needed for these infants since currently most succumb to their disease very quickly despite aggressive chemotherapy.

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APPENDIX

Dosage and Therapy Schedule for ALinC 12 (POG 7623)

	Regimens 1 and 2*	Regimen 3
Number of group 1 (group 2) patients treated†	294 (34)	172 (51)
Phase of treatment		
Induction	V, 2mg/M^2/IV/ wk × 4, P, 60mg/ M^2/day p.o. × 28 and L-asp, 10,000 U/kL/ wk × 2	VP
Consolidation	6MP, 50mg/M^2/day p.o. until CNS therapy complete	L-asp, 6,000 U/M^2/ d × 14 CTX, 1g/ M^2/IV on days 1 and 14, followed by IV MTX, 15mg/M^2/day × 4 q 2 wk × 6
Treatment of CNS subclinical	IT MTX, 12mg/ M^2 28/wk × 5 Cranial RT†	§Triple IT medicine q 2 wk × 6
Continuation treatment (3 yr)	6MP, 50mg/M^2/day p.o. MTX, 20mg/ kL/week p.o.	Same as in Regimen 1
Systemic reinforcement	V, 2mg/M^2/IV/wk × 3 P, 40mg/M^2/ day p.o. × 21	Same
CNS reinforcement	None	§Triple IT, q 8 wk × 3 yr

Ara-C, arabinosyl cytosine; 6TG, 6 thioguanine; CTX, cyclophosphamide; P, prednisone, V, vincristine; L-asp, L-asparaginase; 6MP, 6 mercaptopurine; MTX, methotrexate; IT, intrathecal, q, every; CNS, central nervous system; RT, radiation therapy.

*Regimen 2 was identical to regimen 1 except that the WBC count was kept between 3,000 and 4,500/μL in regimen 1 and between 1,500–3,000/μL in regimen 2. (No difference in relapse incidence has been noted between these patient groups.)

†Only patients receiving all phases of therapy are shown here and in Fig 1.

‡Cranial RT: children >2 years, 2,800 rad; children 1 to 2 years, 2,000 rad; and children <1 year, 1,500 rad.

§Triple intrathecal (IT) MTX 15mg/kL, Ara-C 30mg/kL, hydrocortisone 15mg/kL.

Abnormal Spectrin in Hereditary Elliptocytosis

By Sally L. Marchesi, William J. Knowles, Jon S. Morrow, Marcia Bologna, and Vincent T. Marchesi

An abnormal α subunit of erythrocyte spectrin has been described in hereditary pyropoikilocytosis (HPP), a rare hemolytic anemia characterized by erythrocyte budding and fragmentation. In HPP spectrin, the N terminal domain of the α subunit (α I T80) shows increased susceptibility to tryptic digestion, resulting in cleavage to a 50,000-d peptide, presumably due to a change in primary structure of the α I domain which alters conformation and generates the new cleavage site. The functional result of this conformational alteration is marked impairment of spectrin oligomer formation in vitro, consistent with the established role of α I T80 in spectrin self-association. In the present study, we demonstrate an abnormal spectrin α subunit in two kindreds with hereditary elliptocytosis (HE) that is qualitatively identical to HPP spectrin. Clinical expression of HE in these families ranges from mild elliptocytosis without hemolysis to severe poikilocytic hemolytic anemia clinically resembling HPP. In all affected individuals, a fraction of α I T80 is abnormal, as shown by its cleavage during mild tryptic digestion to the 50 kd peptide described in HPP; the fraction of α I T80 affected is directly proportional to the severity of clinical expression of HE. Spectrin oligomer formation is likewise impaired to a degree which correlates with hematologic disease. One of the HE kindreds studied demonstrated polymorphism in the spectrin α II domain, previously described as a frequent occurrence in blacks. This family also demonstrates a variant α III domain in spectrin that has not previously been described. We conclude that the abnormality in the α I domain originally described in HPP spectrin is shared by a subset of patients with HE; the severity of clinical expression, ranging from mild nonhemolytic HE to poikilocytic hemolytic anemia, is related to the fractional quantity of the α subunit that is affected.

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HEREDITARY elliptocytosis (HE) has long been thought to be a genetically determined disorder of the red cell membrane; however, until recently, there has been no direct evidence to support this hypothesis. Although inheritance of HE occurs in an autosomal-dominant fashion, its expression is extremely variable, the number of elliptocytes in the peripheral smear ranging from <10% to 100%. Compensated hemolysis is common, but significant anemia occurs in only a small number of patients. Poikilocytic variants of HE with severe hemolysis may occur in early childhood in kindreds with elliptocytosis; the peripheral smears of these children show marked microcytosis, pyknocytosis, and fragmentation of erythrocytes.[1-6] In some of these cases, both parents have HE, are consanguineous, or both, whereas in others only one parent has HE or both parents are normal hematologically. Frequently, the severity of the hemolysis ameliorates with age, and the patient develops more typical elliptocytosis with mild to moderate hemolysis.

In 1975, Zarkowsky et al[7] described three children from two kindreds with severe hemolytic anemia characterized by striking poikilocytosis, pyknocytosis, microspherocytes and budding forms. One parent had mild elliptocytosis, whereas the other three parents were normal. Because the morphologic changes in the peripheral blood of the patients resembled that seen in heat injury, heat-induced fragmentation of the patients' cell was studied; it was found that the patients' cells fragmented at 45 °C in contrast to 49 °C for normal cells, and the syndrome was thus termed hereditary pyropoikilocytosis (HPP). Calorimetric studies show a major structural transition in HPP ghosts and spectrin at 45 °C compared with the normal transition at 49 °C.[9] These studies strongly suggested that spectrin was abnormal in HPP.

We and others[10-13] have reported the analysis of erythrocyte spectrins from HPP patients and their relatives, some whom have HE, by two-dimensional isoelectric focussing (IEF) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of intermediate-sized tryptic peptides. These studies have demonstrated an alteration in affected subjects of the *N*-terminal 80,000-dalton domain of the spectrin α subunit (α I T80), which has been shown to bind to dimeric spectrin to promote formation of spectrin oligomers.[14] In a previous study from our laboratory,[10] spectrin from HPP patients and from one sibling with HE showed increased susceptibility of α I T80 to mild tryptic digestion, with production of 50 kd and 21 kd peptides which are not generated in significant amounts from normal spectrin under the conditions used. Similar observations have been made in HE and HPP spectrin by Lawler et al.[12,13] Spectrin from these individuals demonstrates decreased ability to self-associate to form tetramer and higher oligomers, as shown either by an increased content of dimer relative to higher forms in fresh extracts at 4 °C[5,11-13,15] or by impaired concentration-dependent oligomer formation under isotonic conditions at 30 °C.[10] These studies have been interpreted to mean that a molecular alteration (amino acid substitution, deletion) in α I T80, as evidenced by the abnormal susceptibility to tryptic digestion, is responsible for decreasing oligomer stability and consequently decreased stability of the membrane skeleton, resulting in red cell shape change, fragmentation, and hemolytic anemia.

A relationship between HPP and HE is suggested by: (1) the clinical resemblance of HPP to poikilocytic infantile variants of HE, some of which have been thought to represent homozygous HE[1,2,4]; (2) the occurrence of HE in parents or other family members of some patients with HPP[7,10,13,15]

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and (3) a mildly decreased thermal stability of red cells from some kindreds with HE, in which fragmentation occurs at 47 °C to 48 °C.[8,16]

In a strict sense, the term HPP should not be applied to patients with poikilocytic variants of HE unless heat stability studies have been performed; however, these varients of HE (which may be called poikilocytic hemolytic elliptocytosis (PHE)) should probably be grouped with HPP until the specific molecular pathologies producing this clinical picture are better defined.

We report here studies of spectrin from two kindreds with hereditary elliptocytosis; one family of five (a mother and four children) which includes two individuals with compensated hemolysis, and a second family (grandmother, mother, child) with elliptocytosis that is expressed in the child as a severe poikilocytic hemolytic anemia. The spectrin of all affected family members shows an alteration in the N-terminal 80-kd domain of the α subunit (α I T80), which is identical qualitatively to that previously reported for hereditary pyropoikilocytosis, but which involves a smaller fraction of the total α subunit.

MATERIALS AND METHODS

Blood collection, preparation of red cell ghosts and extraction of spectrin. Blood was collected by venipuncture into ACD anticoagulant; informed consent was obtained from all donors and the quantity of blood drawn was appropriate to the estimated blood volume and hematocrit of the subject. Red cell ghosts were prepared by lysis of washed red cells for 30 minutes at 0 °C in 10 to 15 vol of 5 mmol/L of sodium phosphate, 1 mmol/L of EDTA pH 8.0 containing 130 μmol/L of phenylmethylsulfonyl fluoride (PMSF) and 0.1 mmol/L of diisopropyl fluorophosphate (DFP), followed by three washes with the same buffer. Spectrin was extracted by 48-hour dialysis of ghosts at 4 °C into 0.1 mmol/L of EDTA, pH 9.4, containing 130 μmol/L of PMSF and 0.1 mmol/L of DFP. The vesiculated ghosts were removed from the spectrin extract by 2× centrifugation at 48,000 g for 60 minutes at 4 °C. The spectrin extract was then dialyzed into buffer containing 10 mmol/L of Tris, 20 mmol/L of NaCl, 130 mmol/L of KCl, 1 mmol/L of EDTA, 1 mmol/L of β-mercaptoethanol, 30 μmol/L of PMSF, and 0.2 mmol/L of DFP at pH 7.4, and stored on ice.

Tryptic digestion of spectrin. Aliquots (200 μg) of spectrin extract were dialyzed against 20 mmol/L of Tris-HCl pH 8.0, and 1 mmol/L of 2-mercaptoethanol for 20 hours at 4 °C. Trypsin (TPCK-treated, 206 units/mg; Worthington) was added at an enzyme-substrate ratio of 1:20 wt/wt. The reaction was carried out for 90 minutes at 4 °C and was terminated by adding DFP to a concentration of 1 mmol/L and cooling to −80 °C. The digests were lyophilized and stored at −20 °C prior to electrophoresis.

PAGE. Lyophilized spectrin tryptic digest (200 μg) was solubilized in 75 μL of a solution containing 9 mol/L of urea, 2% Triton, 5% β-mercaptoethanol and 2.4% ampholines (LKB Instruments, Inc, Rockville, Md) and focused on 3 × 20 mm polyacrylamide gels according to O'Farrell[17] for 5,700 volt hours. The pH gradient extended from 7.2 to 4.5 and was formed as previously described.[10] After electrophoresis, the gels were equilibrated for ten minutes in 10% glycerol, 3% SDS, 1 mmol/L of EDTA and 2% β-mercaptoethanol before electrophoresis in the second dimension on 10% to 15% acrylamide gradients, using Laemmli buffers.[18]

Analysis of spectrin oligomers. Formation of spectrin oligomers was studied by electrophoresis of extracted spectrin on 2% to 4% gradient nondenaturing polyacrylamide slab gels at 4 °C. The gels were electrophoresed at 50 V for 48 hours in buffer containing 40 mmol/L of Tris, 20 mmol/L of Na acetate, and 2 mmol/L of EDTA pH 7.4.[19] Spectrin extracts prepared at 4 °C as described above were concentrated to 12 to 14 mg/mL by vacuum dialysis against 10 mmol/L of Tris, 20 mmol/L of NaCl, 130 mmol/L of KCl, 1 mmol/L EDTA, 0.5 mmol/L of NaN_3, pH 7.4. Serial dilutions of spectrin in the same buffer were incubated for three hours at 30 °C prior to electrophoresis. In some cases, nondenaturing gels were run in the second dimension on 7% to 12% acrylamide gradients in SDS according to Laemmli.[18]

Quantitation of spectrin oligomers and peptides from Coomassie blue-stained acrylamide gels was performed by elution of excised gel slices with 1.0 mL of 25% pyridine for three to four days until the gel slice was colorless. Optical density at 700 nm of the eluates was measured in a Varian DMS 90 spectrophotometer.

I^{125} peptide maps. Two-dimensional maps of I^{125}-labeled limit chymotryptic peptides were prepared from intermediate-sized tryptic peptides cut from two-dimensional polyacrylamide gels. Na ^{125}I (carrier free, 350 to 600 mCi/mL) was obtained from Amersham (Arlington Heights, Ill). Labeling of excised gel bands was performed by the method of Elder[20] with modifications.[21] Digestion with chymotrypsin and two-dimensional cellulose peptide maps were formed as previously described.[21]

NTCB cleavage. Spectrin was solubilized in 7 mol/L of guanidine, 200 mmol/L of Tris HCl, and 1 mmol/L of EDTA at pH 8.0, and was cleaved at cysteine residues by 2-nitro-5-thiocyanobenzoic acid (NTCB) at a concentration 10 times the cysteine content. After one-hour incubation at RT, the pH was raised to 9.0, and the solution was incubated at 37 °C overnight. The reaction was terminated by addition of 50 mmol/L of β-mercaptoethanol. The spectrin fragments produced were analyzed by two-dimensional IEF/SDS-PAGE and the peptide mapping procedure described above.

Photomicrographs. Wright's-Giemsa stained blood smears were photographed at 200 × magnification on a Olympus BH-2 photomicroscope.

RESULTS

Erythrocyte spectrin obtained from 22 members of seven kindreds with hereditary elliptocytosis has been analyzed by mild tryptic digestion at 0 °C followed by two-dimensional (IEF/SDS) PAGE of the intermediate-sized tryptic peptides produced. When applied to normal spectrin, this technique generates a map of >50 well-resolved peptides with mol wts ranging from 80 kd to 12 kd.[21] Limit chymotryptic mapping of these peptides combined with generation of overlap peptides obtained by enzymatic and chemical cleavage has led to construction of a model of the spectrin heterodimer that includes five major trypsin-resistant domains within the α subunit and four major domains within the β subunit. These are identified in Fig 1. The α I domain is an 80 kd peptide which is relatively resistant to further proteolysis under the mild conditions used (see Fig 1). There is, however, some cleavage of α I T80 to produce a 74 kd peptide just below and at the same IEP as α I T80. The derivation of the 74 kd peptide from α I T80 has been shown by limit peptide mapping and by monoclonal antibody blot.[22] α I T74 is frequently seen in digests of normal spectrin, but is variable in quantity.

Two-dimensional peptide maps of spectrin obtained from three of seven HE kindreds studied were normal except for polymorphism in the α II 46 kd domain (α II T46) previously described in blacks.[23] Spectrin from affected members of two

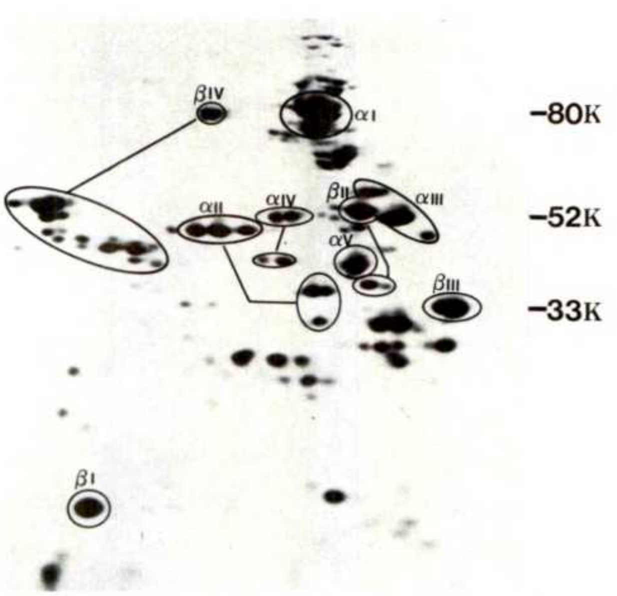

Fig 1. Two-dimensional IEF/SDS polyacrylamide gel electrophoresis of intermediate sized tryptic peptides of spectrin extracted from normal red cells. The major domains of α and β subunits, as defined by mild tryptic digestion, are shown; in some cases, peptides derived from further tryptic cleavage of a parent domain are also shown.

other kindreds with HE showed variably decreased quantities of the 80 kd domain of the α subunit (α I T80) and appearance of a new 50 kd peptide at approximately the same isoelectric point which was previously shown by limit peptide mapping to be derived from the α I 80 kd domain.[10] Concentrated spectrin of affected family members also showed a reduction in oligomer formation of varying degrees when analyzed over a concentration range of ~ 1 mg/mL to 14 mg/mL. These changes are qualitatively similar to those previously reported for patients with hereditary pyropoikilocytosis (HPP), although in no case was all of the α subunit affected as has been seen in HPP.[10] These families are reported in detail below. The remaining two families with HE showed a different structural alteration in the α I domain, which will be reported separately.

Family R. Family R (Fig 2A) is Puerto Rican and consists of a mother and her four children. M.R. Sr is a 46-year-old woman with hematocrits of 36% to 40%, reticulocyte count of 1.3% to 2.1% and peripheral smears showing slight anisocytosis and polychromasia but no elliptocytes. A.R., her 19-year-old son, had hyperbilirubinemia at birth; when he was 6 months of age, his hemoglobin was 9 g/dL, and the smear showed marked elliptocytosis and poikilocytosis. Between the ages of 5 and 17, his hematocrit rose to normal (>40%), and the smears now show typical elliptocytosis. His bilirubin is normal, but haptoglobin is 0 to 10 mg/dL (normal 20 to 200 mg/dL). W.R., 16 years old, has a hematocrit of 42%, reticulocyte count of <1%, some ovalocytic erythrocytes, and a haptoglobin of 31 mg/dL. M.R. Jr, 14 years old, has hematocrits ranging from 33% to 39%, with reticulocyte count of 1.5% to 3%, haptoglobin of 0 and 6 mg/dL, and smears showing marked elliptocytosis. A fourth child, S.R., 12, has a hematocrit of 39%, a reticulocyte count of 0.2%, and a normal smear. The father was not available for study. Peripheral smears from this family are shown in Fig 3A through C.

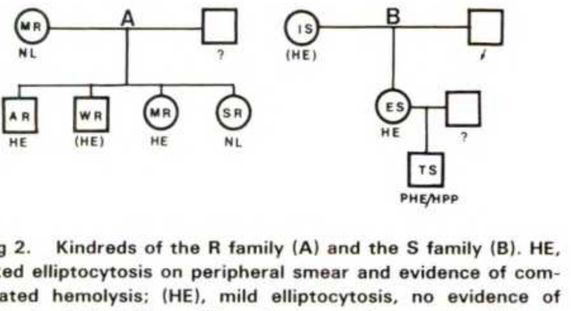

Fig 2. Kindreds of the R family (A) and the S family (B). HE, marked elliptocytosis on peripheral smear and evidence of compensated hemolysis; (HE), mild elliptocytosis, no evidence of hemolysis; PHE/HPP, poikilocytic hemolytic anemia clinically resembling HPP.

Family S. Family S (Fig 2B) is black and consists of a child, his mother, and maternal grandmother. I.S. is a 57-year-old woman with ovalocytes and occasional fragments on smear (Fig 3D). Her hematocrits range from 35% to 40% with reticulocyte count of 1.5%, and a haptoglobin of 77 mg/dL. Her daughter, E.S., 30 years old, had a hematocrit of 24% at one year of age with a reticulocyte count of 5%; the smear showed marked elliptocytosis, anisocytosis, and poikilocytosis. Her hematocrit gradually rose; at 28 years of age, she had a hematocrit of 34% with a reticulocyte count of 2.4% and a haptoglobin of 0. The smear continues to show marked elliptocytosis (Fig 3E) but without the poikilocytosis seen earlier. T.S., the 12-year-old son of E.S., developed hyperbilirubinemia at birth requiring exchange transfusion. His peripheral smears showed marked poikilocytosis, microcytosis, and fragmentation. At 2 weeks of age, his hematocrit was 22% and his reticulocyte count was 6%. Hemoglobin was AA, G6PD was normal, and Coombs test was negative. At 4 months of age, he developed hepatosplenomegaly. He required five transfusions during the first 6 months of life for hematocrits of 15%; at 2½ years of age, he was transfused for a hematocrit of 12% during a viral infection. Between the ages of 4 and 9 years, his hematocrits were stable at 27% with a reticulocyte count of 14% to 19%. He underwent splenectomy at age 10. Since then, his hematocrits have been 33% to 40% with reticulocyte counts of 1% to 3%. $HgbA_2$ is normal but hgbF is 23% and Heinz body prep is negative. The smear continues to show marked elliptocytosis, poikilocytosis, and pyknocytes (Fig 3F), reflected in an MCV of 54 fL. Although the clinical history of T.S. resembles HPP, tests of thermal stability of his red cells were not performed. The father of T.S. was not available for study, and the maternal grandfather is deceased.

Tryptic digests of HE spectrin. Two-dimensional IEF/SDS-PAGE of intermediate tryptic peptides of family R spectrin is shown in Fig 4. The spectrin of M.R. Sr (Fig 4A) is normal, showing a full complement of the α I 80 kd peptide. The spectrins of A.R. and M.R., Jr (Fig 4B and D), the two children with compensated hemolytic HE, show a moderate decrease in quantity of the α I T80 peptide and a marked increase of a 50 kd peptide with several isoelectric points, present in trace quantities in control spectrin (Fig 1)

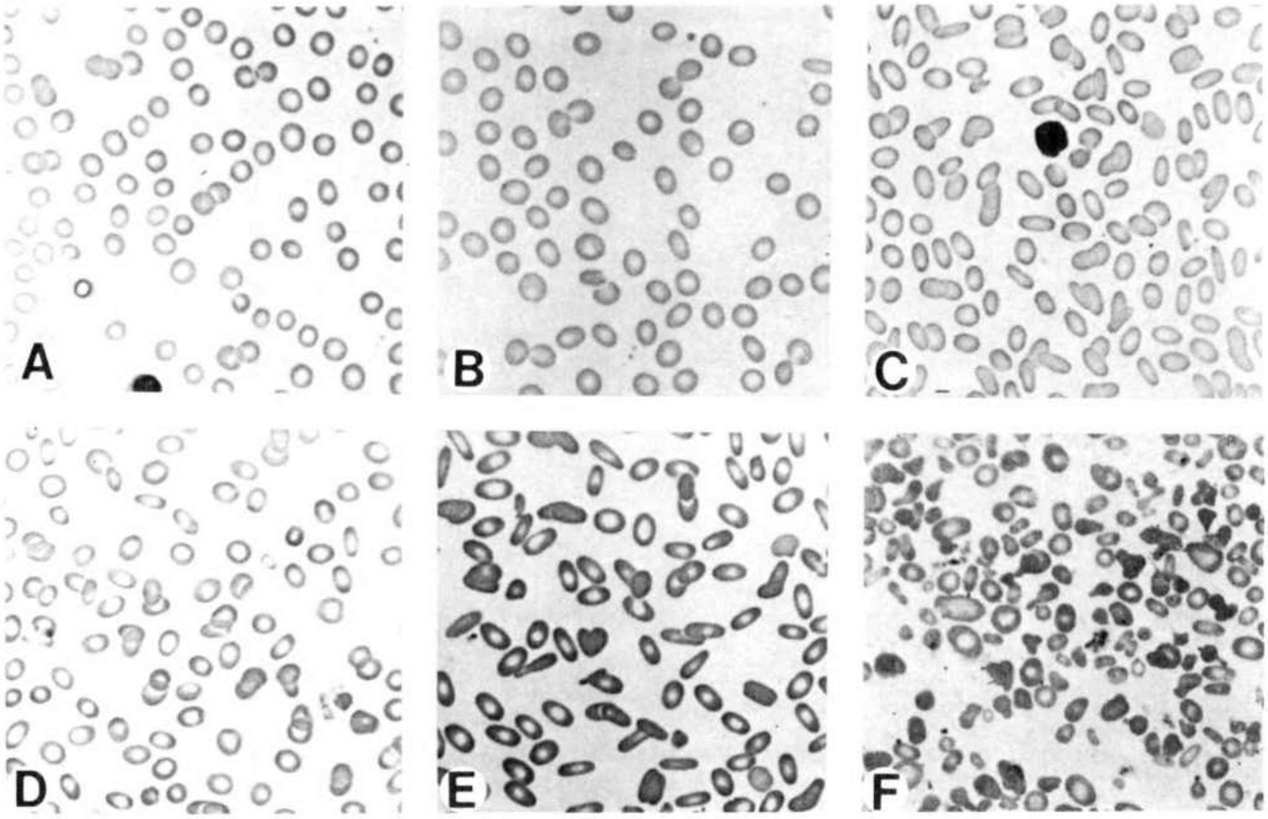

Fig 3. Peripheral blood smears stained with Wright's-Giemsa. R family (A through C): (A) S.R., normal; (B) W.R., mild HE; (C) A.R., hemolytic HE. S family (D through F): (D) I.S., mild HE; (E) E.S., hemolytic HE, (F) T.S., poikilocytic HE (HPP).

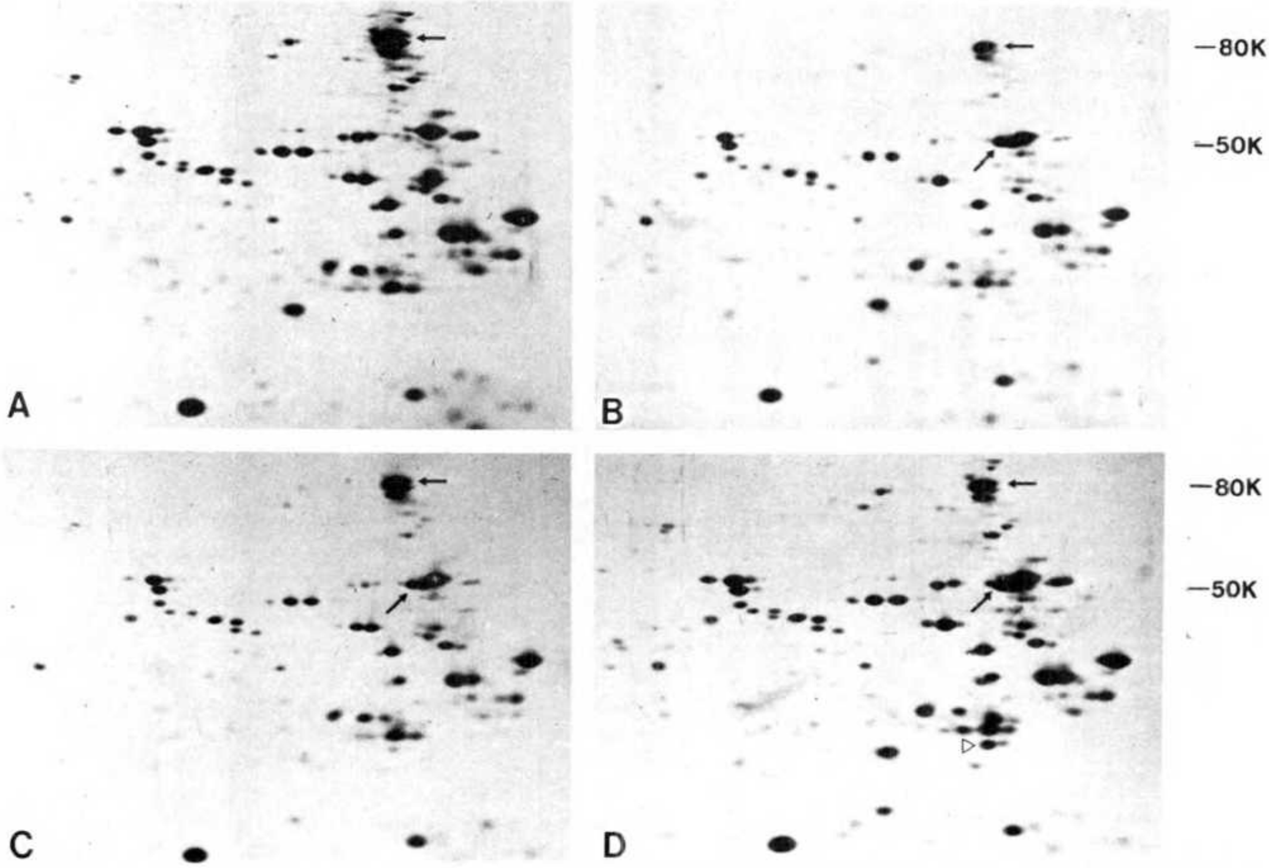

Fig 4. Tryptic peptides of spectrin from members of family R electrophoresed in two dimensions IEF/SDS polyacryamide gel electrophoresis: (A) M.R., Sr, normal; (B) A.R., and (D) M.R., Jr, with marked elliptocytosis and compensated hemolysis; (C) W.R., with ovalocytosis, no hemolysis. Arrows indicate the α I T80 and 50 kd peptides (multiple isoelectric forms). α I T21 (▷) is shown in Fig 4D.

and in the unaffected mother (Fig 4A). W.R., with mildly elliptocytic cells and no evidence of hemolysis, also shows the abnormal α I 50 kd peptide (Fig 4C). Anti-α I T80 monoclonal antibody (23) binds both α I T80 and α I T50 on Western blot of a tryptic digest of AR spectrin (data not shown) consistent with the limit peptide mapping results of Knowles et al,[10] establishing α I T50 as a degradation product of α I T80.

Figure 5 shows the intermediate tryptic peptides of spectrin from family S. I.S. spectrin (Fig 5B) shows a moderate reduction in α I T80, whereas E.S. and T.S. spectrin (Fig 5C and D) show distinct reductions in the quantity of α I T80: in Fig 5D, α I T74 is relatively increased. All three family members show the prominent α I 50 kd peptide observed in affected members of family R.

A second (21 kd) cleavage product of α I T80, identified in limited tryptic digests of spectrin from patients with HPP reported earlier,[10] has been identified by peptide mapping from spectrin digests of members of the R and S families and is demonstrated in Fig 4D and 5B; it can be identified in variable amounts in digests of all affected family members.

The increased susceptibility of the α I 80 kd domain to proteolytic cleavage and the production of the 50 kd and 21 kd peptides observed in these families closely resembles our earlier findings[10] and those of others[11-13] for HPP spectrin, but appears to involve variable percentages of the α subunit. In at least three of the HPP patients reported,[10,12] intermediate tryptic digests showed no residual α I T80, whereas in the R and S families, and in some other patients with HPP,[10,12,13] residual α I T80 is seen in varying degrees. Spectrin peptides α I T80, α I T74, and α I T50 were cut from two-dimensional gels of tryptic digests from the R and S families, and the protein content was quantified by measuring pyridine-eluted Coomassie blue stain (see Methods section). The results are shown in Fig 6, in which α I T50 and α I T74 are expressed as a mole fraction of the total α I

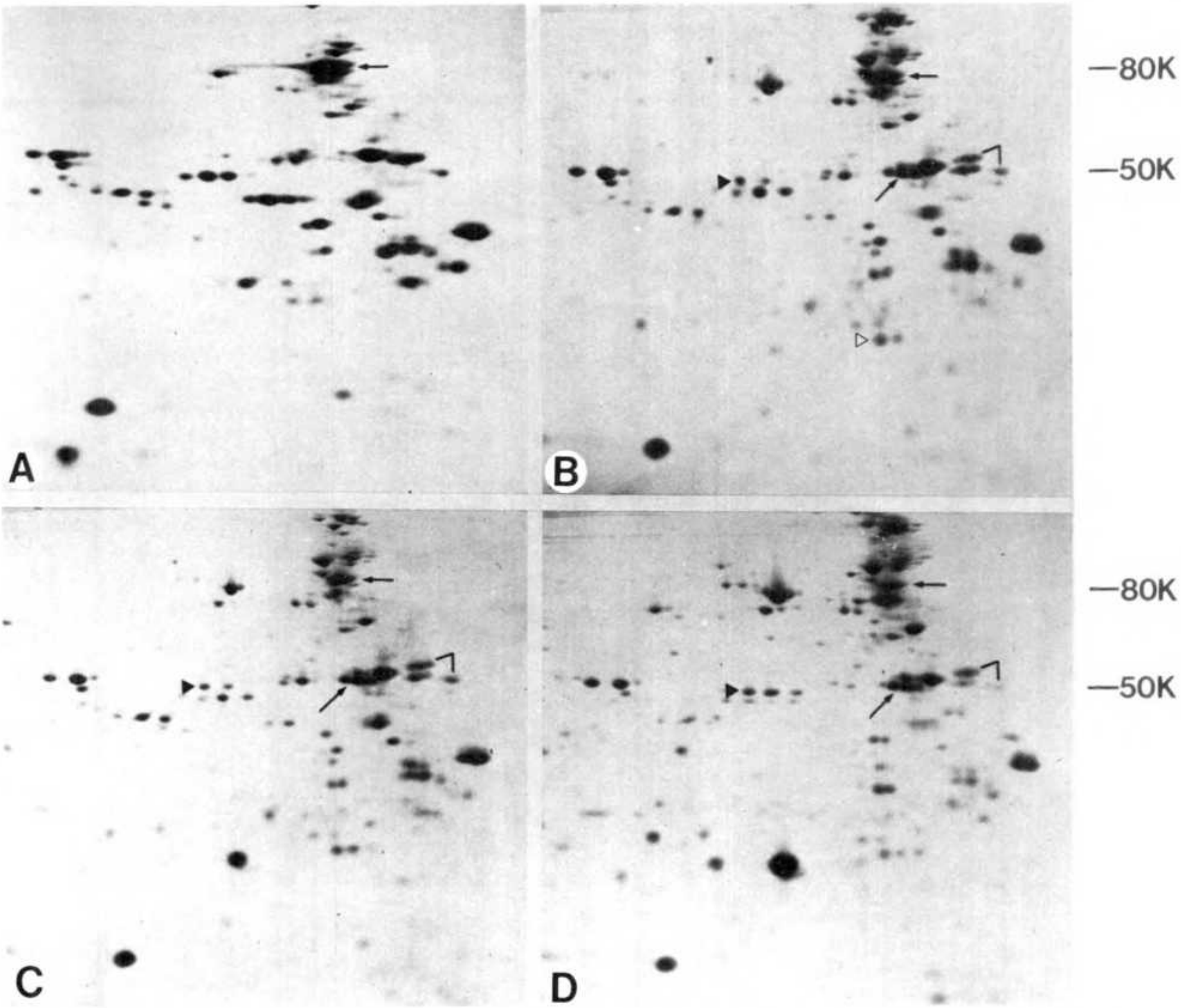

Fig 5. Spectrin tryptic peptides from members of family S electrophoresed in two dimensions IEF sodium dodecyl sulfate polyacrylamide gel electrophoresis: (A) normal spectrin; (B) I.S. with mild elliptocytosis, no hemolysis; (C) E.S. with marked elliptocytosis and compensated hemolysis; (D) T.S. with marked poikilocytosis and hemolytic anemia. Arrows indicate α I T80 and 50 kd peptides in B, C, and D. αIT21 (▷) is shown in Fig 5B. Filled triangles (▶), α II 46 kd domain variants; double pointer (>), α III 52K domain variant. αI T21 (▷) is shown in Fig 5B.

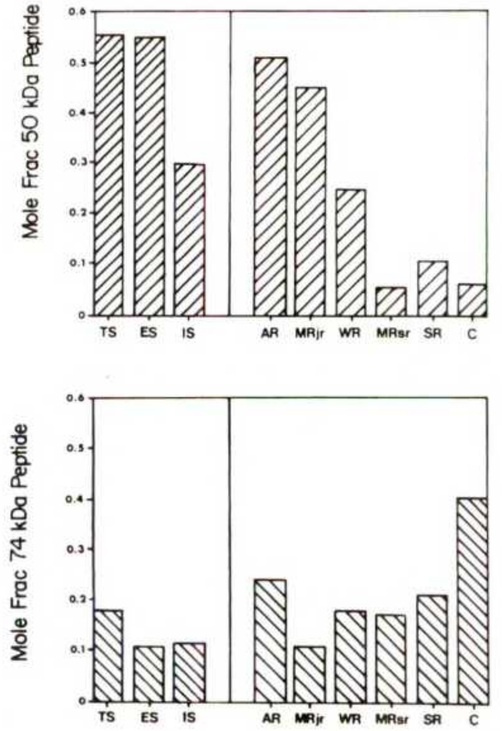

Fig 6. Quantitation by pyridine elution of Coomassie Blue dye of the 50 kd and 74 kd peptides in tryptic digests of spectrin from S and R families, expressed as mole fraction of the total α I T80 domain represented on the gel. Data on T.S. and A.R. are taken from three and two digests, respectively; others are derived from a single digest. Initials of family members are placed in order of decreasing severity of clinical expression of HE for each family.

domain represented on the gel. The initials of the subjects are arranged in order of decreasing severity of hematologic disease within each of the two families. The data shown are taken from one to three two-dimensional gels of spectrin tryptic peptides from each family member and controls. Subsequent quantitation of α I peptides of multiple (three to seven) tryptic digests of spectrin from T.S., E.S., A.R., and controls gave similar results.

It is evident that the mole fraction of α I T50 is highest in the patients from each family with the most severe expression of HE (T.S., E.S., A.R., M.R.) and decreases with decreasing severity of hematologic abnormality. Although α I T74 is increased in some of the tryptic digests of members of these families (as illustrated in 5D [T.S.]), the cleavage of α I T80 to α I T74 is variable in degree in our experience both in normal and HE families. This is shown in Fig 6 (bottom), which expresses the mole ratio of α I T74 to total α I domain peptides from tryptic digests of the S and R families.

Additional α spectrin variants in S family. In addition to the α I T80 abnormality described above, the S family also shows polymorphism in the α II T46 domain of the type previously reported to be common in blacks.[23] In Fig 5A, the α II domain type I migrates as 46 kd peptides at several isoelectric points with cleavage products of 35 kd and 30 kd, and is the type seen in spectrin of all (>100) white subjects studied to date (see also Fig 1). Spectrin of E.S. and I.S. (Figs 5 B and C) shows double heterozygosity for α II T46 types 1 and 2; in type 2, the α II domain migrates as 50 kd, 39 kd, and 35 kd peptides with isoelectric points more basic than α II T46, 35 and 30 (type 1). T.S. spectrin (Fig 5D) is heterozygous for α II T46 types 2 and 3; in the latter, apparent mol wt of α II increases without a change in isoelectric point.

All of the members of the S kindred also show variant α III domain peptides not previously described. A new peptide is seen above and at the same isoelectric point as the normal α III T52 as well as a new α III cleavage product (Fig 5 B through D; see Fig 1). I^{125}-labeled limit chymotryptic maps of the larger peptide (α III T56) are identical to normal α III T52 shown in Fig 7. This α III variant has been seen to date in four other individuals (three kindreds) all of whom are black. Three of these subjects have HE or poikilocytic hemolytic anemia consistent with HPP, and their spectrin shows the α I T80—α T50 cleavage described above, as well as variant α II domains. One of these patients, K.B., was previously reported.[10] Thus, the spectrin of these individuals has alterations in the three of the five domains of the spectrin α subunit.

A number of other differences are apparent on two-dimensional gels of spectrin tryptic digests from normal individuals. These include variability in degree of digestion of the α IV domain and variable intensity of many of the peptides of <30 kd. These differences, which occur randomly, in part reflect differences in protein load, as well as other variables inherent in the experimental technique.

Localization of α I T50 within the α I domain. Cleavage of spectrin at cysteine residues with NTCB followed by two-dimensional IEF/SDS-PAGE yields a complex map of peptides with mol wts between 90 kd and 6 kd. Peptide mapping of some of the NTCB peptides has been performed to identify their location within the domain structure of the spectrin molecule.[21] A 67 kd NTCB peptide has been found by peptide mapping of I^{125}-labeled limit chymotryptic peptides to contain most of the iodinated spots from the α II domain as well as some of the map of α I T50 from HPP spectrin, suggesting that α I T50 is located adjacent to the α II domain. Peptide maps of NTCB 67, α II T46, and α I T50 are shown in Fig 8. The peptide map tracing shows those peptides from α I T50 and α II T46 which are also found in

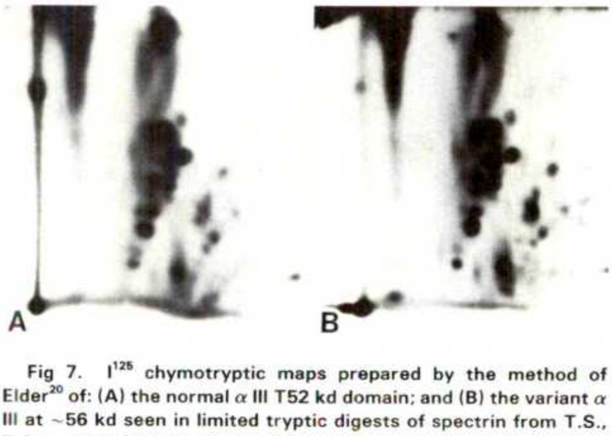

Fig 7. I^{125} chymotryptic maps prepared by the method of Elder[20] of: (A) the normal α III T52 kd domain; and (B) the variant α III at ~56 kd seen in limited tryptic digests of spectrin from T.S., E.S., and I.S. (Fig. 5B through D).

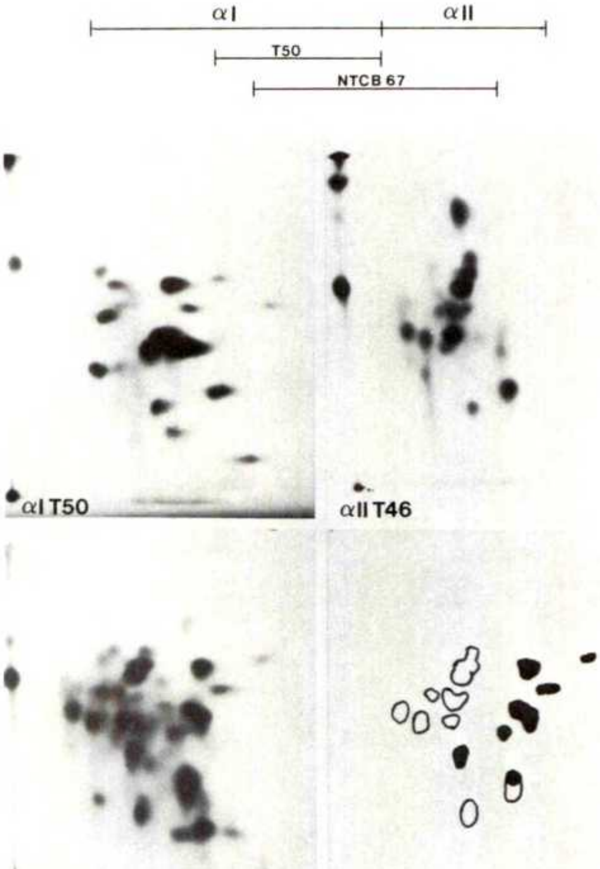

Fig 8. Peptide maps of I^{125}-labeled limit chymotryptic peptides derived from α I T50, α II T46, and NTCB 67 kd peptides. Horizontal axis, electrophoresis; vertical axis, chromatography. The map tracing is a composite of the α I T50 limit peptides (●) and α II T46 limit peptides (○) which appear in NTCB 67. Line drawing at top illustrates the relative locations of the α I and α II domains, α I T50, and the NTCB 67 peptide.

NTCB 67K. These results establish the proximity of α I T50 to the α II domain, as shown in the molecular model at the top of Fig 8. Placement of α I T50 from HPP spectrin in the C terminal half of the α I domain agrees with sequencing data performed on α I T50 derived from normal spectrin (discussed later).

Oligomer formation from HE spectrin. The ability of spectrin heterodimers from family R and family S to form higher oligomers was analyzed by incubation of spectrin at concentrations between 0.8 and 14 mg/mL in isotonic buffer at pH 7.4 for three hours at 30 °C prior to nondenaturing gel electrophoresis. Results are seen in Figs 9 through 11.

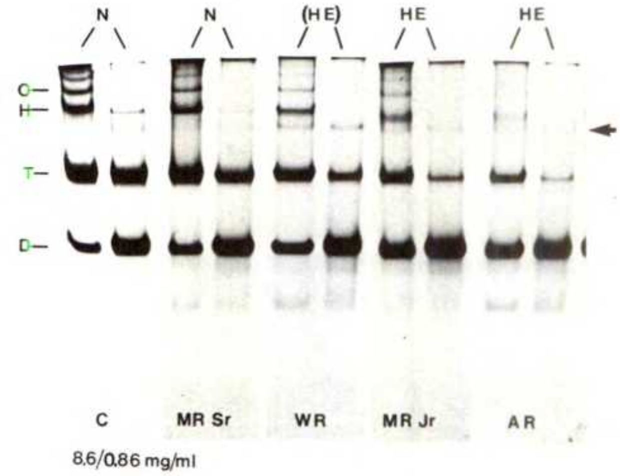

Fig 9. Self-association of spectrin from members of family R. Crude spectrin extracts from family members designated by initials and from a normal unrelated control donor were concentrated and incubated at 30 °C for three hours at 8.6 mg/mL (left lanes) and 0.86 mg/mL (right lanes); 75 μg of each sample was loaded on 2% to 4% acrylamide gels and was run for 48 hours in Na acetate buffer pH 7.4. D, dimer; T, tetramer; H, hexamer; O, octamer. Arrow (right) indicates nonspectrin protein which migrates near hexamer in this gel system. Other nonspectrin proteins are seen migrating ahead of spectrin dimer.

Figure 9 shows oligomer formation by spectrin from the R family at concentrations of 0.86 and 8.6 mg/mL. It can be seen that spectrin from a normal individual (C) and from the unaffected mother, M.R. Sr, when incubated at the 8.6 mg/mL (left lanes) forms higher oligomers, whereas the predominant forms of spectrin at 0.86 mg/mL (right lanes) are dimer and tetramer. Spectrin from A.R. and M.R., Jr, who have compensated hemolytic HE, forms less tetramers than normal at low concentrations and forms less oligomers at high concentration. Spectrin of W.R. (mild nonhemolytic HE) forms oligomers normally at the higher concentration; however, at low concentration, it has a moderately increased dimer-tetramer ratio.

Figures 10 and 11 show oligomer formation by spectrin from the S family. Both T.S. and his mother, E.S., have markedly impaired oligomer formation in vitro over a wide

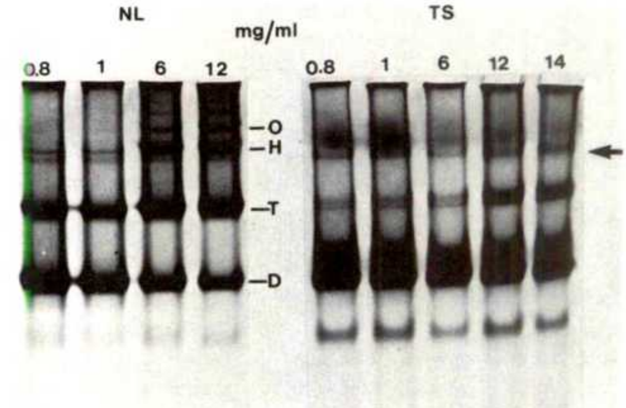

Fig 10. Self-association of normal spectrin and spectrin from T.S. with poikilocytic HE and hemolytic anemia. Crude spectrin extracts were incubated at concentrations from 0.8 to 14 mg/mL at 30 °C for three hours and were then electrophoresed on 2% to 4% nondenaturing acrylamide gels. D, dimer; T, tetramer; H, hexamer. Arrow indicates nonspectrin protein migrating just behind hexamer. Other nonspectrin proteins are seen migrating ahead of spectrin dimer; globin migrates as a diffuse band at the top of the gel.

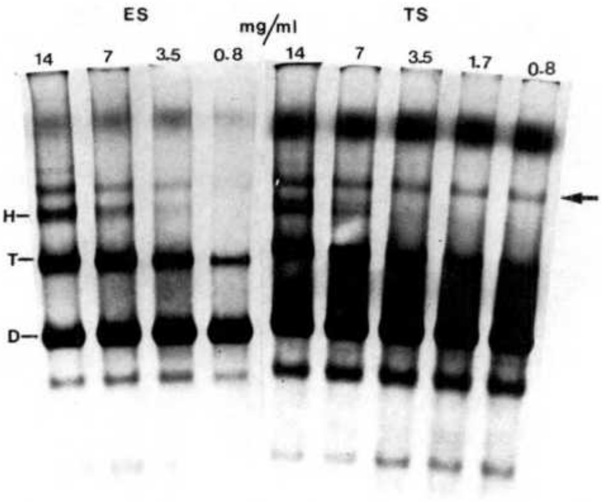

Fig 11. Self-association of spectrin from E.S. (hemolytic HE), and her son T.S. (poikilocytic HE). For details, see legend for Fig 10.

range of concentrations. Crude spectrin extracts from T.S. contain greater than normal amounts of nonspectrin protein, characteristic of spectrin extracts from patients with red cell fragmentation and hemolysis, and include a protein band migrating near spectrin hexamer (arrows). T.S. spectrin also shows smearing of protein between dimer and tetramer; the smear consists largely of spectrin as shown by electrophoresis of the nondenaturing gel into SDS/PAGE in the second dimension (Fig 12). Figure 12 also demonstrates nonspectrin proteins in the crude extract, including a 22 kd peptide (arrow), identified as band 8 (glutathione S transferase)[24] which migrates with the spectrin smear between dimer and tetramer on nondenaturing gels but which separates from spectrin in SDS. No specific interaction between band 8 and the patient's spectrin could be detected by sucrose density gradients of crude spectrin extracts (data not shown).

Quantitative assessment of spectrin oligomer formation in the R and S families is shown in Figs 13 and 14. In Fig 13, the mole fraction of spectrin dimer and of tetramer and higher oligomers is shown for spectrin from family R incubated as described for Fig 9. The initials of family members are again arranged in order of decreasing expression of hematologic disease. In Fig 14, the mole fractions of dimer and tetramer are shown for E.S. and T.S. over the range of spectrin concentrations described in Fig 11; these are compared with a normal control curve derived from a large number of subjects.[19]

It is evident from Figs 13 and 14 that the mole fraction of dimer is elevated and oligomers are decreased in spectrin from all HE/HPP subjects at low spectrin concentrations, and that these differences are not only maintained but are amplified at high concentrations of spectrin; this is important because the spectrin concentration at the membrane surface probably exceeds 100 to 200 mg/mL.[19] These data also show that the degree of impairment of spectrin self-association parallels the hematologic expression of HE and HPP.

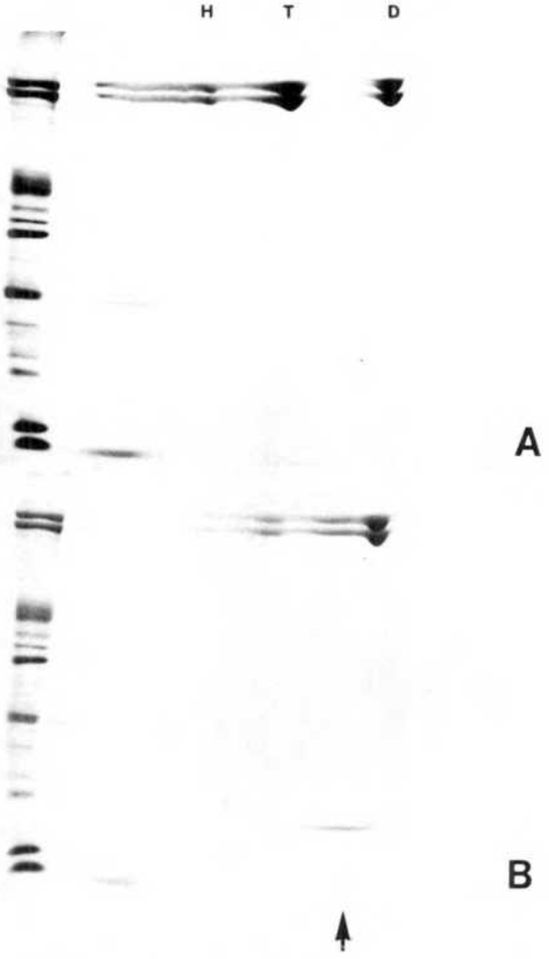

Fig 12. Nondenaturing 2% to 4% acrylamide gels of spectrin from (A) a normal control and (B) a subject which has been electrophoresed in the second dimension into sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 10% to 15% acrylamide gradient. D, dimer; T, tetramer; H, hexamer. Arrow, band 8 (glutathione-s-transferase).

DISCUSSION

HE appears to be a heterogeneous disease caused by deficiencies or abnormalities in several components of the membrane skeleton including, to date, the α and β subunits of spectrin[10–13,15,25,31] and protein 4.1.[26,27] All HE kindreds reported with abnormal spectrin have also evidenced abnormal spectrin self-association, which is thought to be responsible, at least in part, for the shape change, decreased mechanical fragility,[15] and shortened survival observed. We have described here two families with HE whose spectrin demonstrates an abnormal α I domain (α I T80) detected by its increased susceptibility to tryptic cleavage. Normal α I T80 is highly resistant to further tryptic cleavage under the mild digestion conditions used, yielding small (and variable) amounts of a 74 kd peptide and a 50 kd peptide, as well as lower mol wt fragments (S. Marchesi and J. Letsinger, unpublished observations). The α I 74 kd and 50 kd peptides produced from normal spectrin have been shown by Speicher

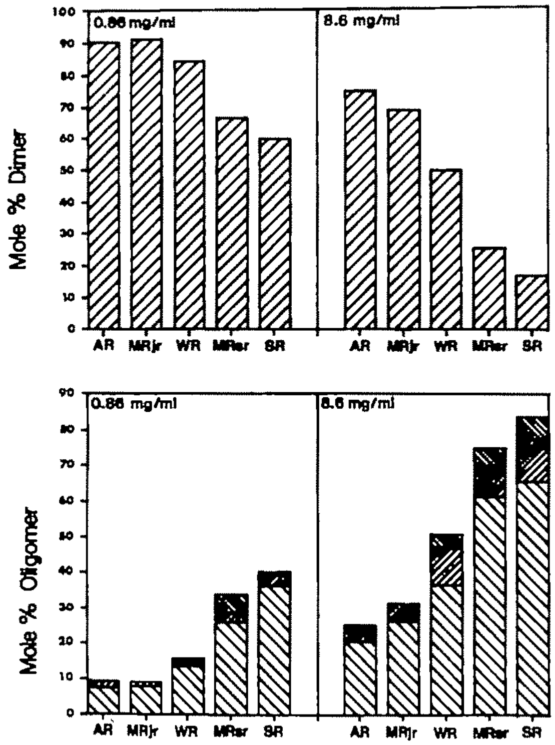

Fig 13. Quantitation of dimer, tetramer, and higher oligomers of spectrin from family R by pyridine elution of Coomassie blue dye stain from native gels shown in Fig 9. See legend for Fig 7 for experimental detail. ▧ tetramer; ▨ hexamer; ▩ octamer.

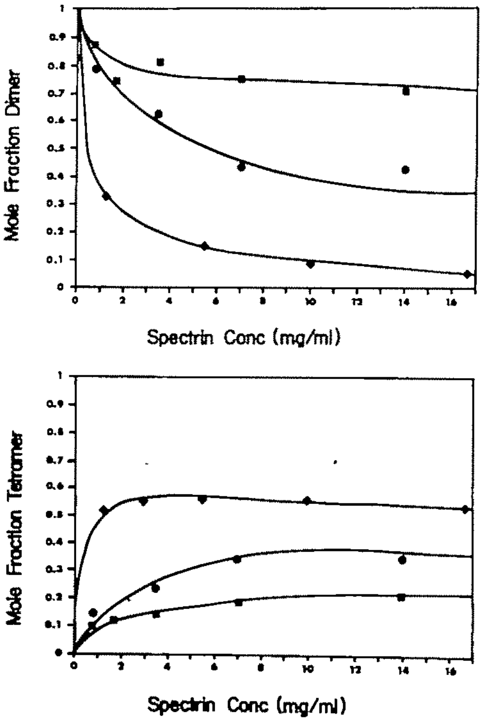

Fig 14. Quantitation of spectrin dimer and tetramer from T.S. (■), and E.S. (●) by pyridine elution of Coomassie blue dye from the native gels shown in Fig 11. See legend for Fig 9 for experimental details. The control curve (♦) is a composite of studies on normal individuals as previously reported.[18]

et al[28] to result from cleavage of peptide from the N terminus of the α subunit. The spectrin of HPP patients is characterized by an α I T80 domain which is readily cleaved under the same conditions to a 50 kd peptide[10] (or 46 kd peptide[11-13]) presumably due to altered conformation of the 50 kd cleavage site leaving little, if any, residual intact α I T80 peptide. By contrast, a smaller fraction of the α subunit is abnormal in patients with HE; whereas two-dimensional peptide maps of all affected individuals demonstrate cleavage of α I T80 to α I T50, a variable amount of unaltered α I T80 remains as shown above and by others.[13]

The cleavage of α I T80 to α I T74, although frequently increased in this population, does not correlate quantitatively with hematologic expression of HE (Fig 6). It should be noted, however, that other investigators have determined the α I 80 → 74 cleavage to be typical of a subpopulation of patients with HPP and HE.[11-13] The differences between their results and ours may be due to methodology, in particular due to characteristics of the digestion conditions including pH and ionic strength, which are known to affect specific cleavage sites (W. Knowles and J. Morrow, unpublished observations).

The most likely interpretation of our observations is that an altered amino acid sequence within the α I domain of HE and HPP spectrin produces conformational changes in neighboring portions of the peptide, resulting in increased exposure of the (normal) tryptic cleavage site at lysine 252,[28] producing an increased quantity of the 50 kd α I peptide, and possibly increasing cleavage at arginine 39 to produce α I T74. The sequence data suggest that both (normal) 50 kd and 74 kd peptides extend to the same COOH terminal site as the intact α I 80 kd.[28] The 50 kd peptide that appears in digests of HE and HPP spectrin is thought to be identical to the normal 50 kd cleavage product, based on its isoelectric point, monoclonal antibody studies, and I^{125} limit peptide mapping which shows only normal peptides of α I 80 kd.

The presumptive alteration in α I primary structure and resultant conformational change is thought to be responsible for the abnormal spectrin–spectrin interactions observed in patients with HE (and HPP) because the α I domain is known to associate specifically with spectrin dimers to promote oligomer formation.[14] Impairment of spectrin self-association has frequently been reported in HPP and HE, expressed either as increased percentage of dimer in crude extracts at 4 °C,[11-13,15,29,30] or as inability to form tetramers and oligomers after incubation at 30 °C over a range of concentrations.[10] Oligomer formation by spectrin from HE subjects in the two families reported here was reduced in all cases; however, the degree of impairment varied from slight in the patients with mild nonhemolytic HE to severe in the case of patient T.S. with poikilocytic hemolytic anemia, approaching our early findings in HPP.[10] As shown in Figs 6, 13, and 14, the severity of clinical expression of HE in members of the kindreds described is proportional to the fractional quantity of α I T80 with altered conformation as well as to the degree that oligomer formation is reduced.

Another possible interpretation of our observations is that the abnormal segment of spectrin is located in the β 1 domain and, because of its interaction with the α I domain, changes in conformation of α I occur which result in an altered tryptic cleavage pattern. However, we have previously demonstrated[10] that tryptic digestion of HPP spectrin which has been denatured and refolded for 60 seconds produces only α I T50 and α I T21 peptides, whereas normal spectrin generates only α I T80. Because the denaturing conditions used results in dissociation of α and β subunits, and because the refolding period was very short, it seems unlikely that the β subunit is responsible for the altered digestion of the α I domain.

Dhermy et al[31] have reported a spectrin β chain variant in a family with elliptocytosis associated with defective self-association of spectrin dimer. No abnormality of the β 1 domain of spectrin from families R and S was seen on SDS-PAGE of intact spectrin nor on two-dimensional maps of tryptic and NTCB digests (data not shown); however, this does not rule out the possibility of an abnormal β 1.

The increases in apparent mol wt of the variant α II and α III domains may be due to insertion of 4 kd or to an amino acid substitution which affects the sites of tryptic cleavage for both domains. Available data on the α II variants do not support these mechanisms for the α II polymorphism (W. Knowles and D. Speicher, unpublished observations). Alternatively, single amino acid substitutions may affect conformation, changing SDS-binding properties and migration on SDS/PAGE, in addition to altering isoelectric point. No information yet shows how expression of the abnormal α I T80 may be modulated by the variant α II and α III domain peptides observed in family S. The α II variants, which are seen frequently in blacks in our studies, confer no observable effect on erythrocyte membrane stability when present alone, but their effect in the setting of an abnormal α I domain is unknown. The α III variant has been seen in three families in the setting of HE and HPP and with α II variants; nothing is known about its effect on the expression of the abnormal α I T80. The only other subject with the α III variant observed to date had HS but an otherwise normal spectrin tryptic digest.

Although HPP appears to be closely related to HE based on family studies, its occurrence within a particular kindred is not predictable. Mentzer et al[15] conclude from a study of two families that HPP results from compound heterozygosity for HE and a nonelliptocytic carrier state characterized by red cell ghosts with increased mechanical fragility. Lawler et al[13] suggest that HPP red cells contain more of a particular abnormal spectrin than HE cells. There may be multiple alleles coding for the spectrin α subunit, as in the α thalassemia syndromes, and with similar consequences. Thus, if members of a family with HE were to have 1, 2, 3, or 4 abnormal alleles coding for the α subunit, a corresponding proportion of spectrin would contain the abnormal α I 80K. As the number of affected alleles increased, the hematologic expression would range from mild HE to severe poikilocytic hemolytic anemia. This concept is particularly attractive because of the multiple α subunit mutations observed in spectrin from the same individual. Thus, variants in α I, α II, and α III seen in the spectrin of one individual need not all occur on the same molecule in the four-allele model. It may be that the α II or α III variants have no pathophysiologic effect if present alone on an α subunit, but if present on the same α subunit as the α I T80 → 50 mutation, they may interact with and modulate expression of hemolytic disease.

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Chronic B-Lymphocytic Leukemia Cells Proliferate and Differentiate Following Exposure to Interferon In Vitro

By Leif Östlund, Stefan Einhorn, Karl-Henric Robèrt, Gunnar Juliusson, and Peter Biberfeld

The ability of interferon (IFN) to induce proliferation and differentiation in malignant B cells from 29 patients with chronic lymphocytic leukemia (CLL) and in lymphoid cells from 11 healthy donors was investigated. IFN induced transformation and plasmacytoid differentiation in B cells from 19 of 29 CLL patients. The transformed cells belonged to the malignant clone as indicated by the immunoglobulin (Ig) light chain restriction. Cells exposed to IFN expressed intracellular Ig to a varying degree, which was correlated to the level of plasmacytoid differentiation. IFN gave rise to proliferative responses in cells from three patients. Cytogenetic studies on lymphoid cells from one patient showed that proliferation occurred in the malignant B cells. Induction of proliferation and differentiation was observed with various α-IFN and γ-IFN preparations, as well as with a completely pure β-IFN, showing that IFN and not contaminants in the preparations were responsible for the observed effects. Maximal transformation and proliferation usually occurred after four days of incubation at an IFN concentration of 500 to 5,000 U/mL. The ability of IFN to induce differentiation in CLL cells may be of importance for the reported antitumoral effects observed in some B cell malignancies during IFN therapy.

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INTERFERON (IFN) can induce remissions in leukemias[1-3] and other malignancies.[4,5] However, the mechanism behind these antitumor effects is not known. It has been suggested that IFN may act indirectly, by stimulating functions of the immune system.[6] IFN may also act directly, by inhibiting cell multiplication,[7,8] and/or by induction of differentiation in the malignant cells.[9,10] Because differentiation blockage is a basic defect leading to malignant clonal expansion, an abrogation of this blockage may lead to a transformation of malignant cells into more benign phenotypes. We now show that α-IFN, β-IFN and γ-IFN can induce blast transformation and differentiation of varying degrees in leukemic cells from ~65% (19 of 29) of patients with CLL as well as proliferation in 3 of 29 patients.

MATERIALS AND METHODS

Patients

Twenty-nine patients with a diagnosis of chronic B lymphocytic leukemia were investigated. Hematological findings, clinical data and Rai stage[11] at the time of sampling are presented in Table 1. Diagnosis was established by morphology and by immunological studies on peripheral blood, bone marrow, and lymph node samples according to the Kiel Lymphoma Study Group.[12] Most patients (22 of 29) had not received any previous treatment for their disease.

IFN and Mitogen Preparations

Natural α-IFN (α-IFN) provided by Dr D. Secher, was prepared from Sendai virus-induced human Namalwa cells and was purified with an anti-IFN antibody affinity system.[13] The specific activity of this preparation was 2 × 10^8 U/1 mg protein, and the purity was ~90%.

Recombinant α_2-IFN (R. α_2-IFN) derived from *E coli*, with a specific activity of 3.2 × 10^8 U/1 mg protein was provided by Dr G. R. Adolf, Ernst-Boehringer-Institut, Vienna. The purity of this preparation was >99%.

Recombinant γ-IFN (R. γ-IFN) with a specific activity of 3.5 × 10^7 U/1 mg protein was provided by Dr G. R. Adolf, Ernst-Boehringer-Institut.

Pure β-IFN was provided by Dr E. Knight, Central Research and Development Department E. I. du Pont de Nemours and Co. This IFN preparation, with a specific activity of ~2 × 10^8 IFN U/1 mg protein, was produced, purified, and characterized as previously described.[14] According to amino acid analysis, it contained no polypeptides other than IFN.[15]

The antiviral activities of the IFN preparations were tested by an assay measuring cytopathogenic effect of vesicular stomatitis virus in fibroblasts.[16] The various IFN preparations were titrated against international standard preparations.

Mock α-IFN was provided by Dr K. Cantell (Finnish Red Cross, Helsinki). It was prepared from buffy coat suspensions of leucocytes using normal allantoic fluid as inducer.[17] It contained no measurable antiviral activity at the concentrations used.

Lipopolysaccharide (LPS), derived from *E coli* (055:B5), was provided by Professor Tord Holme, Department of Bacteriology, Karolinska Institute, Stockholm; it was used at a final concentration of 100 μg/mL.

Phytohemagglutinin M (PHA) (DIFCO Laboratories, Detroit). The substance was rehydrated by adding 5 mL of sterile distilled water, and the solution was used at a final dilution of 1:67.

Cells and Culture Conditions

Heparinized peripheral blood was centrifuged on a Ficoll-Isopaque density gradient.[18] The interface was collected and washed three times in modified Eagle's medium (MEM) (Flow Lab, Irvine, England) supplemented with 10% heat-inactivated human AB serum and antibiotics (penicillin 50 μg/mL, streptomycin 50 μg/mL). A sample of the cells was stained with crystal violet solution and counted. A final concentration of 2 × 10^6 lymphocytes per milliliter was used. Ten-milliliter cultures for studies on cellular morphology and cellular immunoglobulin content were set up in 50-mL plastic flasks (Falcon 3031, Oxnard, Calif). For measurement of proliferative response, 0.2-mL microcultures were set up in round-bottomed 96-well plates (Flow Lab). Cultures were then incubated for one to six days in a humidified atmosphere with 5% CO_2 at 37 °C. Mitogens and IFN preparations were present throughout the cultures. The cell viability was determined by trypan blue staining. Usually, between 1 and 2 × 10^6 cells per milliliter remained after

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Table 1. Clinical Data

Patient	Age	Sex	Diagnosis	Rai Stage	Duration of Disease (yr)	Previous Treatment	Hemoglobin (g/L)	Lymphocyte Count ($\times 10^{-9}$/L)	Platelet Count ($\times 10^{-9}$/L)	Surface Membrane Immunoglobulin Phenotype
1	38	F	CLL	I	5	No	129	300	259	μdk
2	60	F	IC	IV	10	No	111	50	70	μk
3	72	M	IC	I	5	No	131	32	161	μdk
4	58	M	IC	II	4	Yes	151	20	280	μdk
5	59	M	CLL	I	5	No	136	110	140	μdk
6	82	M	CLL	II	5	No	137	200	187	μλ
7	65	F	CLL	I	4	Yes	139	14	152	μdk
8	56	M	CLL	I	3	No	158	20	303	μdλ
9	82	M	IC	I	2	No	105	17	124	μdλ
10	69	M	CLL	0	1	No	155	21	218	μλ
11	56	M	CLL	I	2	No	143	15	118	μdk
12	61	M	CLL	I	1	No	129	34	160	μdλ
13	65	M	CLL	I	3	No	168	200	124	μdk
14	84	F	CLL	I	1	Yes	125	32	258	μdλ
15	64	F	CLL	IV	3	Yes	132	36	284	μλ
16	62	F	CLL	I	0.5	No	127	35	180	μdk
17	50	F	CLL	0	1	No	134	18	282	μdk
18	81	M	CLL	I	5	Yes	111	68	114	μdk
19	72	M	IC	II	1	No	109	152	103	μdk
20	68	F	IC	I	0.5	No	117	72	242	γk
21	48	M	IC	0	1	No	162	10	248	γk
22	72	M	IC	IV	8	Yes	136	20	20	μλ
23	72	F	CLL	I	0.5	No	110	100	242	μdλ
24	75	M	CLL	III	13	No	87	200	215	μdk
25	81	F	CLL	IV	5	Yes	119	39	119	μdλ
26	57	M	CLL	0	0.5	No	157	27	168	μk
27	68	M	IC	0	0.5	No	165	28	220	?*
28	65	M	CLL	I	2	No	147	15	197	μλ
29	69	F	CLL	0	0.5	No	148	13	266	μdk

*Staining too weak to permit identification.

four days in culture. With few exceptions, the proportion of dead cells in these cultures varied between 1% and 5%.

Assay for DNA-Synthesis

Twenty-four hours before harvesting, 1.0 μCi of ^{3}H-thymidine with a specific activity of 5 Ci/mmol (TRA 120, Radiochemical Centre, Amersham, England) was added to each culture. Harvesting was performed with a Skatron Harvesting Machine (Lierbyen, Norway) and the radioactivity was measured with a scintillation spectrophotometer (Packard Instrument Company, Inc, Downers Grove, Ill). These assays were performed in triplicate. The variation within triplicates was usually <15%. Induction of proliferation was defined as an increase in counts per minute (cpm) of >100% above medium control as well as a numerical net increase of $\geq 2 \times 10^3$ cpm.

Measurement of Morphological Changes

If not otherwise stated, morphological changes were evaluated after four days of culture. Approximately 1×10^5 cells in 0.1 mL were centrifuged (Cytospin, Shandon Southern, Runcorn, England) for ten minutes onto glass slides, and were then stained with May-Grünewald-Giemsa (MGG) and mounted with cover glass in Eukitt. The samples were examined "blindly" in light microscope. Because there was a considerable variation in the size of primary cells as well as cells cultured in medium without stimulants, we classified the cells into four groups as follows[19]: SL, small lymphocytes (<12 μm); SB, large lymphocytes and small blasts (12 to 15 μm); MB, medium blasts (15 to 19 μm); and LB, large blasts (>19 μm). At least 200 cells were counted and the percentage of each lymphocyte subgroup was determined. The data were scored as follows: SB, 1 point; MB, 2 points; LB, 3 points. To allow a statistical evaluation of the morphological transformation, a Lymphoblast Transformation Score (LTS) formula was created: LTS = (SB% × 1) + (MB% × 2) + (LB% × 3). A net increase in the LTS by 20 points or more was arbitrarily designated as positive morphological transformation. This LTS index correlated well with the subjective impression of transformation in individual clones.

Immunofluorescence and Peroxidase–antiperoxidase (PAP) Technique

After 5 days in culture, the cells were washed three times in balanced salt solution (BSS) and were then incubated at 37 °C for one hour in BSS. Cytospin preparations were fixed in acetone for ten minutes at 4 °C. Surface immunoglobulin (Ig) was demonstrated by immunofluorescence after incubation with fluorescein isothiocyanate (FITC) conjugated F(ab′)$_2$ rabbit antibodies to human Ig of light κ and λ chains (Kallestads Lab, Chaska, Minn).[20] Intracellular Ig (κ and λ light chains) was demonstrated by the PAP-technique[21,22] with suitable monoclonal antibodies.

Chromosome Analysis

Cytogenetic analysis was performed on cells from patient 3, cultured with and without 5,000 U/mL of α-IFN for four days. After treatment with colchicine and a hypotonic potassium chloride solution, the cells were fixed in methanol/acetic acid and were then air-dried as previously described.[23] Q-banded metaphases suitable for chromosome analyses were photographed in a Zeiss fluorescent microscope and were analyzed by conventional karyotyping from paper copies.[24]

Statistical Analyses

Statistical differences were evaluated using Student's *t* test (number of patients > 20) or the Wilcoxon matched-pairs signed-ranks test (number of patients < 20). Pearson correlation coefficients were used to evaluate correlations.

RESULTS

Induction of Blast Transformation

α-IFN at the concentration 5,000 U/mL transformed the lymphocytes to blasts in cultures from 19 of 29 patients (LTS increase of more than 20 points) (Fig 1). By means of a concentration of 50 U/mL, blast transformation was observed in lymphoid cells from 10 of 25 patients (Table 2, showing data from the first tests performed). The mean increase in LTS using α-IFN was statistically significant for both IFN concentrations ($P < .001$). Five thousand IFN U/mL was more efficient in inducing blast transformation than was 50 U/mL ($P < .05$). There was also a good correlation in induction of transformation between these two IFN concentrations ($P < .001$). LPS induced transformation in cells from 18 of 29 patients (Table 2). There was a close correlation between IFN-induced and LPS-induced transformation (50 and 5,000 U/mL; $P < .001$). Lymphoid cells from four responding patients were tested on four different occasions within one year. Lymphocytes from three of these patients responded to IFN by blast transformation at all times. In cells from one patient, transformation occurred on two occasions, whereas no effects were observed in the other two instances (data not shown). Cells from four patients not responsive to IFN were also tested at several occasions; no responses were seen at any test (data not shown). These findings indicate that responsiveness to IFN, with some

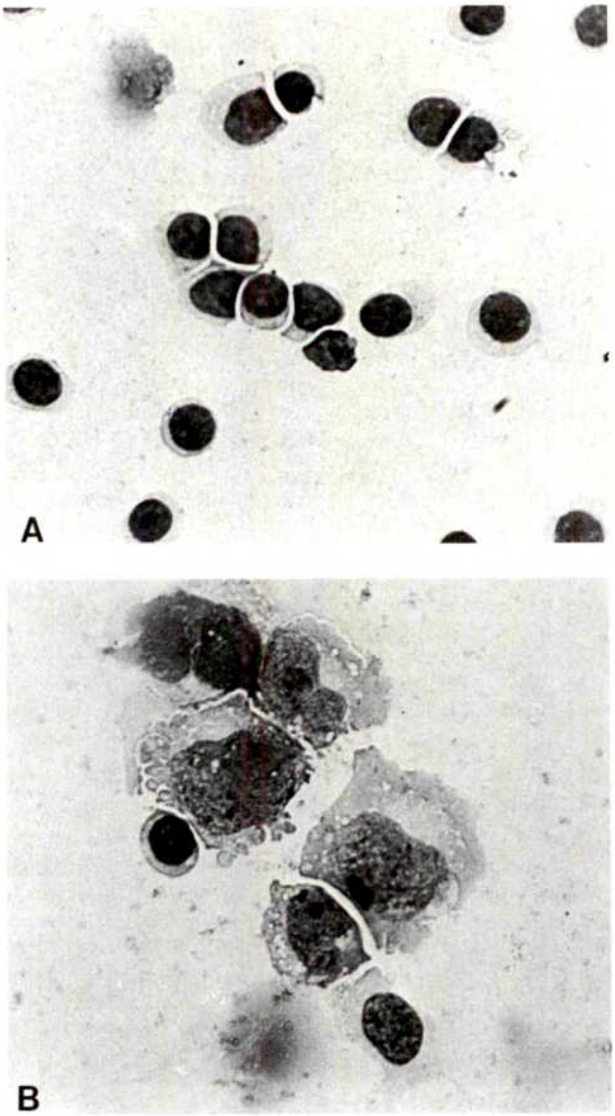

Fig 1. Lymphoid cells from patient 3 cultured for four days: (A) without IFN, showing a homogeneous picture of small regular lymphocytes; and (B) with α-IFN 5,000 U/mL, showing a varying degree of morphological transformation into blasts and plasmacytoid cells.

Table 2. Lymphoblast Transformation in Lymphoid Cells From 29 Patients and 11 Healthy Donors Cultured for 4 Days In Vitro Together With α-IFN, LPS, or Medium Only

Patient	Medium	IFN (50 U/mL)	IFN (5,000 U/mL)	LPS
1	59	117	141	136
2	3	8	4	4
3	31	33	62	63
4	6	ND	30	48
5	25	73	71	81
6	6	6	14	17
7	27	ND	56	34
8	20	43	46	87
9	8	65	61	54
10	12	11	18	26
11	3	4	5	8
12	47	91	82	148
13	52	59	83	54
14	13	ND	58	71
15	36	74	96	105
16	2	7	10	47
17	17	60	87	58
18	13	63	54	72
19	65	78	98	84
20	5	69	83	78
21	21	15	38	44
22	35	23	82	41
23	24	33	26	26
24	10	36	18	24
25	15	22	32	35
26	17	8	46	46
27	10	16	34	11
28	26	ND	16	65
29	12	23	46	38
Patients	22 ± 3	43 ± 6	52 ± 6	56 ± 6
Controls	9 ± 3	ND	12 ± 3	15 ± 14

Data are expressed in Lymphoblast Transformation Score (LTS); mean ± SE for patients and controls are shown.

LPS, lipopolysaccharide; ND, not done.

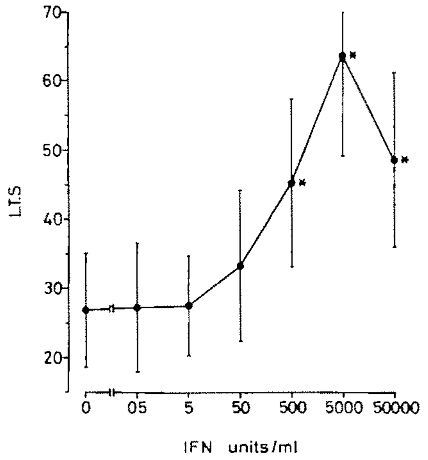

Fig 2. Lymphoblast transformation in malignant B cells after four days of incubation in varying concentrations of α-IFN. Means ± SE from eight patients. Data are expressed in Lymphoblast Transformation Score (LTS); *$P < .05$.

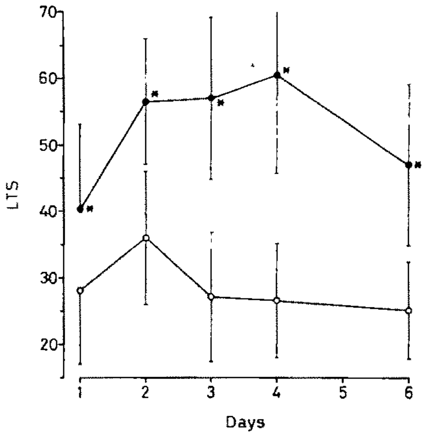

Fig 3. Lymphoblast transformation in malignant B cells after exposure to medium without (O—O) or with 5,000 U/mL of α-IFN (●—●) for one to six days. Means ± SE from seven patients. Data are expressed in Lymphoblast Transformation Score (LTS); *$P < .05$.

exceptions, is a fairly constant characteristic of a malignant clone.

Lymphocytes from 11 healthy donors showed no major increase in blast transformation after four days of culture with 5,000 U/mL of α-IFN (Table 2). Cells from one donor responded to LPS with blast transformation.

Surface immunofluorescence (κ and λ Ig chains) was studied in IFN-responsive cells from four patients. Between 95% and 100% of the cells exposed to IFN expressed membrane light chains of one type only, demonstrating that most of the cells undergoing blast transformation were clonal.

Dose of IFN and Lymphoblast Transformation

Lymphoid cells from eight patients were incubated with 0 to 50,000 U/mL of α-IFN for four days. In lymphocytes from six of the patients, IFN induced blast transformation. The maximal transformation was observed at IFN-concentrations of 500 to 50,000 U/mL (Fig 2). Induction of blast transformation was statistically significant for the three highest IFN concentrations used ($P < .05$).

Kinetics of IFN-Induced Lymphoblast Transformation

Lymphoid cells from seven patients were incubated with or without 5,000 U/mL of α-IFN for 1, 2, 3, 4, and 6 days (Fig 3). After one day in culture with α-IFN, the cells showed a significant increase in the degree of blast transformation ($P < .05$). The IFN-induced transformation seemed to be most pronounced after four days in culture (Fig 3).

Differentiation of Transformed Cells Detected With PAP-Staining

In most cases, a proportion (30% to 80%) of IFN-transformed CLL cells showed a plasmacytoid morphology. These cells, included in the SB group and MB group, were characterized by a relatively dense chromatin and by smaller nuclei than those of the other transformed cells. The plasmacytoid level of differentiation of malignant cells was confirmed by intracellular immunoglobulin κ- and λ-light chain staining. An increased proportion of IFN-transformed cells displayed intracellular immunoglobulin of the same light chain isotype as that of the surface membrane Ig phenotype (Table 3).

Induction of Proliferation

In lymphoid cells from 3 of the 29 patients tested (patients 3, 9, and 27) 5,000 U/mL of α-IFN increased the uptake of ^{3}H-thymidine after four days in culture (Table 4, data from the first tests done). The proliferative responses to IFN in cells from these patients were confirmed in repeated experiments during the course of the disease. Cells from these three patients were also responsive to LPS, and cells from 93% (26

Table 3. Differentiation in Cells From Patients 1 and 3

		Surface Light Chains				Intracellular Light Chains							
	Cultured	κ		λ		κ				λ			
Patient	With:	o	+	o	+	o	+	++	+++	o	+	++	+++
1	No IFN	0	100	97	3	90	9	1	0	100	0	0	0
	IFN	0	100	95	5	62	29	8	1	99	1	0	0
3	No IFN	0	100	100	0	100	0	0	0	100	0	0	0
	IFN	0	100	100	0	90	5	5	0	100	0	0	0

Cells were cultured for four days with 5,000 U/mL of α-IFN or medium only. PAP-staining was used to detect κ and/or λ light chains in or on transformed cells. According to degree of staining, cells were divided into the following groups: o, not demonstrable; +, weak; ++, moderate; +++, strong. Data are expressed in percentages.

Table 4. Incorporation of ^{3}H-thymidine (cpm × 10^{-3}) in Lymphoid Cells From 29 Patients and 11 Healthy Donors

Patient	Medium	IFN (50 U/mL)	IFN (5,000 U/mL)	LPS	PHA
1	3.1	4.2	4.6	1.8	4.0
2	1.1	1.0	0.6	1.5	86.0
3	3.5	12.8	49.2	54.1	119.2
4	0.8	0.5	0.9	0.6	48.1
5	0.5	0.5	0.6	0.5	1.8
6	0.4	0.4	0.4	0.5	6.4
7	0.8	0.8	0.7	2.3	100.2
8	0.4	0.5	0.4	2.2	29.3
9	1.3	2.1	5.3	4.0	28.8
10	0.6	0.5	0.5	0.6	38.8
11	0.6	0.5	0.4	1.5	22.5
12	3.0	1.2	3.1	3.3	30.8
13	0.6	0.3	0.4	0.4	2.9
14	1.3	1.1	0.6	2.4	57.2
15	1.9	1.9	0.8	2.1	39.6
16	0.2	0.1	0.3	0.3	31.8
17	0.7	1.0	1.4	0.6	ND
18	0.4	0.6	0.4	0.5	19.3
19	0.3	0.1	0.3	0.3	23.0
20	1.3	1.1	1.1	3.2	19.1
21	0.7	1.0	0.5	1.2	22.0
22	0.6	0.5	0.7	1.4	20.3
23	0.3	0.3	0.6	0.6	28.0
24	0.4	0.4	1.0	0.9	5.6
25	0.6	0.7	0.9	1.3	19.8
26	0.8	1.0	1.1	2.1	41.8
27	1.3	6.4	6.1	3.7	152.5
28	2.0	2.8	3.8	3.9	17.0
29	0.5	1.0	0.5	1.8	50.8
Patients	1.0 ± 0.2	1.6 ± 0.5	3.0 ± 1.7	3.4 ± 1.8	38.1 ± 6.7
Controls	1.2 ± 0.2	ND	1.3 ± 0.3	1.1 ± 0.3	34.1 ± 2.5

Cells were cultured for four days in vitro together with α-IFN, LPS, PHA, or medium only. Mean ± SE for patients and controls are shown.
LPS, lipopolysaccharide; PHA, phytohemagglutinin; ND, not done.

of 28) of all patients were responsive to phytohemagglutinin (PHA) (Table 4).

Lymphoid cells from 11 healthy donors were cultured together with α-IFN. No major increase in ^{3}H-thymidine uptake was observed after exposure of the cells to IFN. Cells from one donor responded to LPS, and lymphoid cells from all donors responded to PHA by proliferating.

Dose of IFN and Proliferation

Lymphoid cells from 9 patients (3 with cells responsive and 6 with cells nonresponsive to 5,000 U/mL of α-IFN) were incubated with 0 to 50,000 U/mL of α-IFN for 4 days (Fig 4). In responsive cells from the three patients, maximal stimulation of proliferation occurred at 500 to 5,000 U/mL of α-IFN. At the highest IFN concentration used (50,000 U/mL), the proliferative response was less pronounced. In nonresponsive cells from the six other patients, none of the IFN concentrations gave rise to a proliferative response.

Kinetics of IFN-induced Proliferation

Lymphoid cells from eight patients (3 with cells responsive and 5 with cells nonresponsive to 5,000 U/mL in 4-day cultures) were incubated for 1, 2, 3, 4, and 6 days in the presence or absence of 5,000 U/mL of α-IFN (Fig 5). In responsive cells from the three patients, maximal proliferation occurred after three to four days of incubation. In nonresponding cells from the five other patients, no IFN-induced proliferation was observed on either 1, 2, 3, 4, or 6 days of incubation.

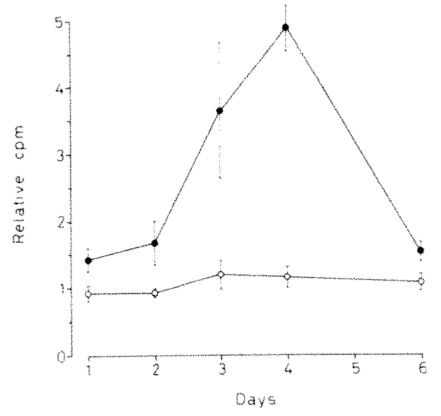

Fig 4. Incorporation of ^{3}H-thymidine in IFN-responsive cells from three patients (●—●) and in cells not responsive to IFN from six patients (○—○) (means ± SE). Cells were cultured in the absence or presence of 5,000 U/mL of α-IFN for one to six days. Relative cpm = cpm with IFN divided by cpm with medium only.

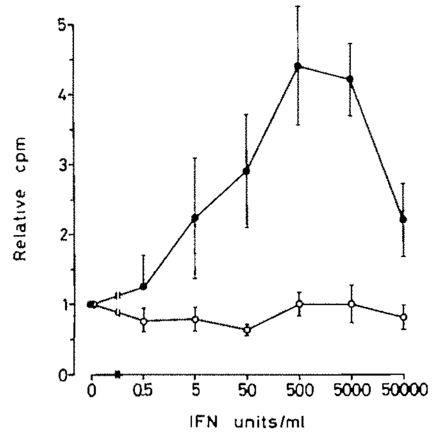

Fig 5. **Incorporation of ^{3}H-thymidine in IFN-responsive cells from three patients (●—●) and in cells not responsive to IFN from six patients (○—○) (mean ± SE). Cells were cultured for four days with different concentrations of α-IFN. Relative cpm = cpm with IFN divided by cpm with medium only.**

Proliferative Responses to Combinations of IFN, LPS, and PHA

To study the influence of IFN on LPS-induced and PHA-induced proliferation, cells from eight patients were incubated with LPS or PHA in the absence or presence of α-IFN (Table 5). Lymphoid cells from three patients responded to IFN by proliferating. The addition of both IFN and LPS to cell cultures from these patients did not give rise to an additive effect on proliferation. With the highest IFN concentrations used, there was a tendency toward an inhibition of the proliferative response (Table 5). In the cell cultures that did not respond to IFN alone by proliferating, addition of IFN had no major influence on the LPS-induced proliferative responses. Addition of IFN to PHA-stimulated cultures caused an inhibition of the proliferative responses (Table 5).

Table 5. Incorporation of ^{3}H-thymidine (cpm × 10^{-3}) in Lymphoid Cells

Substance (U/mL)	Response to IFN (n = 3)	No Response to IFN (n = 5)
Medium	2.0 ± 0.8	2.7 ± 0.9
IFN (50)	6.9 ± 3.1	4.0 ± 1.6
IFN (5,000)	20.3 ± 14.5	3.2 ± 1.4
IFN (50,000)	20.3 ± 10.8	3.8 ± 2.0
LPS	22.1 ± 16.0	10.7 ± 3.3
LPS + IFN (50)	34.5 ± 19.9	7.9 ± 3.0
LPS + IFN (5,000)	17.6 ± 11.3	10.0 ± 3.4
LPS + IFN (50,000)	11.7 ± 4.9	8.3 ± 3.1
PHA	88.7 ± 29.9	52.1 ± 31.0
PHA + IFN (50)	84.0 ± 31.2	60.1 ± 28.0
PHA + IFN (5,000)	54.4 ± 22.0	35.6 ± 18.5
PHA + IFN (50,000)	37.6 ± 18.0	25.7 ± 15.9

Cells were cultured for four days in vitro with α-IFN (50, 5,000, or 50,000 U/mL) and/or LPS or PHA. Data are presented as mean ± SE. Cells from all patients responded to LPS and PHA. Cells from three of the patients responded to IFN.

LPS, lipopolysaccharide; PHA, phytohemagglutinin.

Table 6. Transformation in Lymphoid Cells From Seven Patients

Patient	Medium	Mock α-IFN	α-IFN	R.α_2-IFN	Pure β-IFN	R.γ-IFN
3a	31	27	62	58	66	ND
3b	27	19	49	ND	75	ND
3c	6	ND	29	30	75	ND
3d	5	ND	27	ND	ND	25
3e	44	ND	97	ND	ND	ND
4	6	ND	28	ND	36	ND
9	11	ND	78	ND	43	ND
22	12	ND	54	ND	ND	43
26	17	ND	46	ND	43	ND
27a	9	9	67	41	32	38
27b	10	ND	34	ND	36	ND
29	12	ND	46	55	ND	ND

Cells were cultured for four days in vitro with 5,000 U/mL of different IFN preparations. Data are collected from several experiments (a, b, . . .) and expressed in Lymphoblast Transformation Score (LTS).

ND, not done.

Cytogenetic Analysis on Induced Cells

Chromosome analysis was carried out in cells from patient 3. Fifteen metaphases from the cell culture stimulated with α-IFN (5,000 U/mL) and nine metaphases in LPS-stimulated cells were studied. In each of these cell cultures, three metaphases had an identical chromosomal aberration. The aberrant chromosome was a no. 11 with the long arm deleted at band q22, as previously reported (patient 117 in ref. 23). The remaining metaphases all had a normal 46,XY karyotype. Unstimulated cell cultures yielded no evaluable metaphases.

Studies Using α-IFN, β-IFN and γ-IFN Preparations

Different IFN preparations of varying purities were tested for their capacity to induce proliferation and transformation in CLL cells. Lymphoid cells from the patients were incubated with pure β-IFN, natural α-IFN, recombinant α_2-IFN, recombinant γ-IFN, and mock α-IFN (Tables 6 and 7). The results showed that all IFN preparations, including the pure β-IFN, induced proliferation and blast transformation in the malignant B lymphocytes, whereas the mock-IFN preparation had no effect. There seemed to be no major difference in the relative capacity of α-IFN, β-IFN, and γ-IFN to induce proliferation and blast transformation (Tables 6 and 7). Neither did lymphoid cells that showed no response to α-IFN by proliferation and/or blast transformation show any response to β-IFN or γ-IFN.

Table 7. Proliferative Responses in Lymphoid Cells From Patients 3 and 9

Patient	Mock α-IFN	α-IFN	R.α_2-IFN	Pure β-IFN	R.γ-IFN
3a	ND	12.1	ND	ND	3.9
3b	ND	14.1	ND	ND	ND
3c	0.9	4.7	3.0	ND	ND
3d	0.7	3.5	2.1	3.7	ND
9	ND	2.8	ND	3.5	ND

Cells were cultured for four days with 5,000 U/mL of different IFN preparations. Data are collected from several experiments (a, b, . . .) Incorporation of ^{3}H-thymidine is presented as relative cpm = cpm with IFN divided by cpm with medium only.

ND, not done.

Correlation Between Clinical Data and Transformation/Proliferation

No correlations between α-IFN–induced transformation/differentiation and clinical parameters, such as hematological data or clinical stage were found. IFN-induced proliferation was detected in three untreated males with the diagnosis immunocytoma (IC) (patients 3, 9, and 27), with moderate increases in lymphocyte counts and Rai stages 0 through 1. Patients with high lymphocyte counts showed lower responses to PHA stimulation ($P < .05$).

DISCUSSION

An enhancing effect of IFN on mitogen-induced differentiation of normal and malignant B cells has previously been reported.[9,10,25] We now demonstrate that leukemic cells from a large number of patients with chronic B lymphocytic malignancies respond to IFN in vitro with blast transformation and with differentiation to plasmacytoid cells without the addition of other stimulatory agents. Membrane immunofluorescence studies showed that most blast transformed cells were monoclonal B cells. Studies of morphological changes as well as the induction of intracellular Ig light chains showed that varying proportions of the leukemic cells transformed into plasmacytoid levels of differentiation following exposure to IFN.

The observed effects were not due to contaminants in the IFN preparation used, since the results were similar using several IFN preparations, one of which was completely pure. Maximal effects were usually observed after four days in culture and at IFN concentrations of 500 to 5,000 U/mL. These IFN concentrations are found in the serum of patients receiving IFN by the intravenous route.

In most of the patients, blast transformation was not accompanied by a measurable induction of proliferation, as tested at different times using a range of IFN concentrations (Figs 4 and 5). This suggests that DNA synthesis is not required for IFN-induced blast transformation to occur. In cells from three IC patients, however, IFN induced a proliferative response, measured by an increase in the ^{3}H-thymidine uptake. The clonality of the proliferating cells was evidenced by the finding that in one case metaphase cells contained a clonal chromosomal aberration. Stimulation of tumor growth in vitro by IFN has previously been suggested by others.[26,27] However, IFN is generally known as an inhibitor of cell proliferation.[5,7,8]

The individual response patterns to IFN-induced proliferation and transformation were usually reproduced in repeated experiments during the course of the disease. It has previously been demonstrated that a battery of B cell mitogens can be used to classify leukemic lymphocytes into various subsets with different functional and clinical characteristics.[28,29] The mechanism behind and the physiological significance of the IFN-induced transformation and proliferation is poorly understood. However, we have recently studied the effect of IFN on enriched B lymphocytes from healthy donors. These data suggest that IFN can also stimulate certain normal B cell subsets and that stimulation of proliferation is not T cell dependent.[30]

In another recent study, we have shown that leukemic B cell clones that respond to IFN by blast transformation also express enhanced levels of the enzyme 2′-5′oligoadenylate synthetase on exposure to IFN, whereas this enzyme was not induced in CLL clones that did not respond to IFN by blast transformation. The observed correlation between induction of 2′-5′oligoadenylate synthetase and blast transformation, indicates that some CLL clones do not differentiate following exposure to IFN because the cells have a resistance to the action of IFN.[31]

IFN has been shown to induce remissions in some patients with B cell malignancies. The mechanisms behind these effects are not known. A previous investigation in tumor patients showed that of several tested immunological functions only natural killer cell activity increased during α-IFN therapy.[6] However, the increments of natural killer cell activity were not correlated to the response to treatment in myeloma patients.[32] IFN-induced differentiation, by means of an abrogation of the maturational arrest in tumor cells, may play an important role in the antitumoral action of IFN in leukemias. Furthermore, studies on the effect of IFN on leukemic cells in vitro may constitute an experimental system for the identification of patients in whom antitumor effects of IFN treatment can be expected.

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HTLV-III Infection After Bone Marrow Transplantation

By Joseph H. Antin, Brian R. Smith, Bruce M. Ewenstein, Robert J. Arceci, Jeffrey M. Lipton, Peter L. Page, and Joel M. Rappeport

We prospectively documented the development of a fatal, secondarily acquired severe immunodeficiency in a 19-year-old man who underwent uncomplicated bone marrow transplantation. He had no graft *v* host disease (GVHD) and had normal recovery of his immune system as determined by lymphocyte phenotyping, mitogenic responses of his peripheral blood lymphocytes, and his ability to secrete immunoglobulin. This alteration in immunity was associated with the acquisition of antibody to HTLV-III. His only risk factor for the development of HTLV-III infection was the transfusions he had received during the transplant and recovery period. Two of his 54 transfusions were from an asymptomatic individual at high risk for acquired immunodeficiency syndrome (AIDS), who was subsequently found to be seropositive for anti-HTLV-III and from whom HTLV-III was isolated. The loss of immunocompetence in patients without chronic GVHD disease is unusual, and our data support the view that this patient's immunodeficiency was due to HTLV-III. When bone marrow transplant recipients without chronic GVHD develop late opportunistic infections, consideration should be given to transfusion-associated AIDS.

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THE ACQUIRED immunodeficiency syndrome (AIDS) is a disease which is thought to be due to a T lymphotropic retrovirus, HTLV-III.[1] It is most often seen in homosexual or bisexual males, intravenous drug abusers, severe hemophiliacs using lyophilized clotting factor concentrates, and individuals from underdeveloped countries in whom the disease is prevalent in the heterosexual population. Occasionally, it occurs in recipients of blood product transfusions.[2-5] Bone marrow transplant recipients receive frequent transfusions, but evidence of HTLV-III infection has not been reported in these patients. Following transplantation, these patients often have persistent defects of cellular and humoral immunity; however, in the absence of chronic graft *v* host disease (GVHD) late opportunistic infections and the loss of reestablished immune function are uncommon.[6-13] We had the opportunity to observe prospectively the recovery and subsequent loss of immune function in a bone marrow transplant patient who was exposed to blood from an individual who is at high risk for AIDS, who was shown to be seropositive for anti-HTLV-III antibody, and from whom HTLV-III was subsequently isolated. The loss of immune function was associated with the development of clinical and laboratory abnormalities that support the diagnosis of HTLV-III infection and associated immunodeficiency.

MATERIALS AND METHODS

All studies were done in accordance with institutional review board guidelines.

Phenotype analysis. Peripheral blood mononuclear cells were obtained by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation of heparinized peripheral blood. Monocytes were depleted by incubation with carbonyl-iron (Technicon, Tarrytown, NY), Ficoll-Paque centrifugation, and plastic adherence. The surface phenotypes were established by incubating the cells for 30 minutes at 4 °C with the fluoresceinated monoclonal antibodies Leu-4, Leu-2, and Leu-3 (Becton Dickinson, Mountain View, Calif) and were washed three times with phosphate-buffered saline (PBS). They were analyzed on a FACS Analyzer (Becton Dickinson, Sunnyvale, Calif).

Lectin-stimulated proliferation. Peripheral blood was collected in preservative-free heparin (100 U/mL) and the mononuclear cells were separated on a Ficoll-Paque gradient. The mononuclear cells were washed twice in Hanks' balanced salt solution (HBSS) (Grand Island Biological Co, Grand Island, NY) and resuspended in RPMI 1640 (GIBCO) with 5% heated inactivated human AB serum, L-glutamine, penicillin, streptomycin, and mycostatin. Blastogenic responses to phytohemagglutinin (PHA) and concanavalin A (Con A) (Difco Laboratories, Detroit) were determined in triplicate in a microtiter system with 10^5 cells per well. Optimal concentrations of mitogens were determined in preliminary experiments on normal donors for each batch of mitogen and, in the case of PHA, three concentrations of PHA over a tenfold range were assessed for each separate patient determination to assure study at maximum response. Median concentrations required were 2 μg/mL of PHA and 3 μg/mL of Con A. Cells were incubated at 37 °C in a 5% CO_2 incubator and were pulsed on day 3 for 18 hours with 1.0 mCi/mL ^{3}H-thymidine (New England Nuclear, Boston, Mass). Cultures were harvested on an automatic microtiter harvester (MASH II; M.A. Bioproducts, Bethesda, Md), and incorporated radioactivity was determined in a scintillation counter. PHA and Con A stimulation indices are determined by dividing the observed counts per minute (cpm) by the background control values; in our laboratory, normal stimulation indices are >20.

Screening of blood donors. Blood donation numbers of all blood products transfused to the patient were provided to the regional blood collecting facility. Donors were contacted by the collecting facility and 15 agreed to complete an extensive questionnaire and to be screened with complete blood counts, lymphocyte phenotyping, and anti-HTLV III serology (performed at the Centers for Disease Control).

RESULTS

Case report. A 19-year-old, white man without prior history of homosexuality, drug abuse, bleeding disorder, travel to Haiti, or transfusion was admitted to the Brigham and Women's Hospital in September, 1981 with acute

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monocytic leukemia (FAB M5a). Complete remission was achieved with daunorubicin and cytosine arabinoside (ara-C), and he received four cycles of consolidation chemotherapy (daunorubicin, ara-C, and 6-thioguanine). During the period of anti-leukemia chemotherapy, he received 10 units of washed red blood cells (RBC), 3 units of single donor pheresis platelets, but no fresh frozen plasma or granulocyte transfusions.

In April 1982, he underwent allogeneic bone marrow transplantation from his HLA-matched, MLR-nonreactive brother. The patient was prepared for transplantation with ara-C 500 mg/m^2/d for seven days, cyclophosphamide 60 mg/kg/day for two days, and 850 rad single-fraction total body irradiation at a midline dose rate of 5 rad per minute. After transplantation, he received four doses of methotrexate (10 mg/m^2) on days 1, 3, 6, and 11. His course was uncomplicated; he had > 500 granulocytes per microliter at day 34, and developed grade I acute GVHD, which did not require therapy. No chronic GVHD was observed clinically or on skin biopsy. Following his transplant, he received 7 units of washed RBC as well as platelet transfusions from 18 different donors, either as platelet pheresis products or as platelet concentrate (prepared from whole blood donors). He never required granulocyte transfusions or fresh frozen plasma. An arteriovenous shunt (which had been placed prior to transplant for vascular access) was removed on day 240 (month 8, October 1982); during the surgery, he received an additional 2 units of washed RBC and two bags of pooled platelet concentrates from a total of 13 donors. The next year was spent primarily at home, with limited contact with nonhousehold members. He had mild persistent thrombocytopenia after transplant, with platelet counts ranging from 70,000 to 100,000/μL. During this time in vitro lymphocyte function returned to normal (Fig 1) as did immunoglobulin levels, but his absolute lymphocyte count had started to decline. He denied homosexual activity or intravenous drug use.

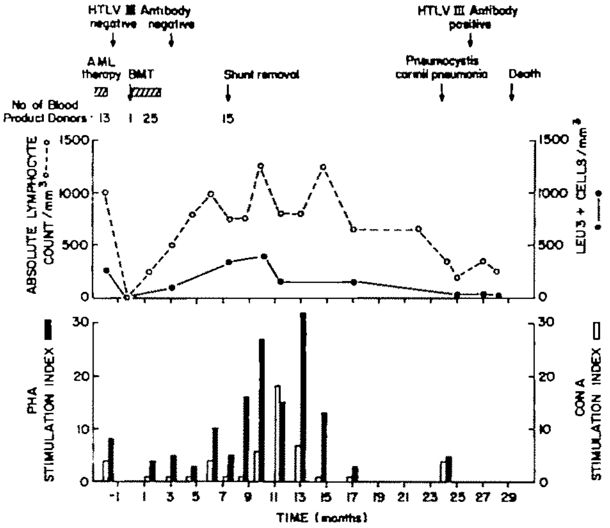

Fig 1. Serial immunologic studies in a patient who developed HTLV-III infection after bone marrow transplantation. There is a progressive improvement in lymphocyte numbers and the mitogenic responsiveness of the patient's lymphocytes to lectins until month 7, ~16 months after the suspected transfusions, when there was a decrease in the numbers of circulating lymphocytes, especially Leu-3+ helper/inducer cells. The reduction in the number of Leu-3+ cells was followed over the next two to four months by a diminution in his blastogenic response to lectins. *P carinii* pneumonia developed ~32 months after the suspected transfusion. In our laboratory, normal stimulation indices are > 20. The hatched areas denote the time periods in which blood products were administered.

On day 680 (month 23, February 1984) he developed a low-grade fever and cough. Over the ensuing two to three weeks, he had slowly progressive shortness of breath; he became hypoxic, and his chest X-ray demonstrated a diffuse interstitial pneumonitis. Open lung biopsy established the diagnosis of *P carinii* pneumonia, and he was successfully treated with intravenous trimethoprim-sulfamethoxazole (TMP-SMX). The TMP-SMX therapy was complicated by the development of severe thrombocytopenia (platelets < 20,000/μL), and therapy was completed with pentamidine. Concomitantly, it was noted that he had developed bilateral cervical lymphadenopathy and severe lymphopenia (200 to 490/μL), and lymphocyte surface phenotyping revealed a marked reduction in Leu-3+ (T4 or "helper/inducer") cells (14/μL), and a "helper/suppressor" (Leu-3/Leu-2) T cell ratio of 0.3. Lymphocyte function testing revealed that mitogenic responsiveness to PHA and Con A was now minimal (Fig 1), and he had no delayed hypersensitivity to intradermal mumps, *Candida*, or tuberculin. Serum immunoelectrophoresis revealed that he had developed a polyclonal increase in IgA (425 mg/dL). Antibody to the Epstein-Barr virus was detected both before transplant and immediately after transplant at 1:640, and it increased to 1:2560 at the onset of the interstitial pneumonia. Cytomegalovirus antibody titer was < 1:8 on both occasions.

After recovery from the pneumonia, he continued to have daily fevers to 101 °F, although no source of infection could be determined. The thrombocytopenia never resolved (range 10,000 to 50,000/μL), although bone marrow aspiration demonstrated adequate numbers of megakaryocytes and showed no evidence of recurrent leukemia. He became severely cachectic, and lost > 20% of his body weight; he did not respond to parenteral nutrition therapy. Over the next three months, he developed recurrent bilateral pneumothoraces, which required virtually constant chest tube drainage. There were no skin lesions consistent with Kaposi's sarcoma. Just prior to his death, he became obtunded and had focal seizures. CAT scan revealed an enhancing lesion in the left frontal cortex, which was thought to be consistent with toxoplasmosis, lymphoma, or abscess. Brain biopsy was refused. He died 850 days after transplantation. Postmortem examination was refused.

HTLV-III. Frozen serum specimens were obtained from March 1982 (immediately prior to the transplant), and day 75 (May 1982, immediately following the transplant) as well as a fresh serum specimen on day 780 (month 25, May 1984). His serum was examined for anti-HTLV-III antibodies by indirect immunofluorescence on an HTLV-III–infected H9 cell line by Essex by an established technique,[14] and he was found to be negative before transplant on day 75 but to be positive on day 780 (Fig 1). The positive test was confirmed by radioimmunoprecipitation (Western blot analysis). The marrow donor had no risk factors for AIDS. He was also seronegative for HBsAg and cytomegalovirus (CMV), and had normal responses to lectin stimulation of his peripheral blood lymphocytes immediately prior to the marrow harvest. Although the marrow donor could not be tested for anti-HTLV-III antibodies, the survey of blood product donors revealed one male, homosexual subject who donated two pheresis platelet products in October and November 1981, both of which were transfused into this patient. At the times of donation, the donor met all of the criteria for normal voluntary blood and platelet pheresis donors. In January 1985, this donor was contacted, and blood was subsequently found to be anti-HTLV-III positive by the Centers for Disease Control; HTLV-III virus was recovered. His complete blood count (CBC) (including absolute lymphocyte count and platelet count) and immunoglobulin levels were normal, but his helper/suppressor ratio was 0.37 (normal 0.9 to 3.5). At the time of writing, he remains asymptomatic.

DISCUSSION

Although the recovery of immune function after bone marrow transplantation may be prolonged, most patients who do not have chronic GVHD have relatively normal in vitro parameters of immune function by 1 year.[10,11] Once in vitro immune reactivity recovers, it generally persists. Furthermore, patients without chronic GVHD rarely develop *P carinii*, or other opportunistic infections as late (> 1 year) complications of transplantation.[12] At a time when immune function is usually stable, this patient developed severe lymphopenia, loss of Leu-3+ lymphocytes, cutaneous anergy, loss of his in vitro mitogen responsiveness, opportunistic infection, diffuse lymphadenopathy, severe thrombocytopenia, and polyclonal hypergammaglobulinemia (IgA). This discrepancy from the usual course of immunologic reconstitution after marrow grafting suggested that the illness was not due to a late complication of transplantation. The clinical syndrome was consistent with the diagnosis of HTLV-III–associated immunodeficiency or AIDS. Because we perform lymphocyte function studies on our transplant patients during and after transplantation, we were able to document prospectively the timing and course of immunologic recovery and subsequent immunoincompetence which led to his death. It is the loss of his in vitro cellular immune reactivity following recovery that led us to suspect that he had AIDS, rather than the persistent defects in cellular immunity that have been reported after marrow grafting. All of the above observations are consistent with the selective infection of Leu-3+ cells with HTLV-III and the consequent disproportionate loss of helper/inducer cells.

His only known risk factor for the development of HTLV-III infection was the transfusion of 54 blood products that he had received during the leukemia treatment, transplant, and recovery period, 2.5 to 3 years prior to his illness and, in particular, 2 units from an anti-HTLV-III seropositive, homosexual individual transfused 16 months prior to the first demonstrable reduction in Leu3+ cells, and 34 months prior to death. The latency between this transfusion and the development of opportunistic infections is consistent with the incubation period observed in transfusion-associated AIDS.[2] It is unusual to be able to document the probable date of HTLV-III exposure and complement that dating with serial immune function studies. It is notable that the patient was seronegative immediately prior to his transplant. The subsequent development of HTLV-III antibodies suggests continued viremia followed by antibody production when his humoral immune response recovered. On the other hand, viremic but antibody-negative patients have been reported,[15] and it is unknown to what extent his underlying leukemia and chemotherapy might have delayed or prevented the development of antibody. However, it is also possible that the blood product that resulted in the HTLV-III transmission was administered either during the transplant or shunt removal from a donor who was not contacted.

The appearance of anti-HTLV-III antibody after transplantation demonstrates that the patient was capable of mounting a humoral immune response to new antigens, and supports the hypothesis that he had actually developed AIDS. Antibodies to HTLV-III are uncommon in the general population, and he received plasma only in association with platelet transfusions; thus, it is unlikely that the antibody titer reflects passive transfer of immunoglobulin.[16–18]

In patients who have undergone marrow grafting and in other patients with immunodeficiencies, abnormalities of the helper/suppressor (Leu-3/Leu-2) T cell ratio are difficult to interpret. Evidence from multiparameter flow cytometric analysis of posttransplant lymphocytes shows that in many patients the Leu-2+ cells may not be mature suppressor/cytotoxic T cells by conventional criteria (ie, simultaneously expressing the surface antigens Leu-2 and Leu-4). Leu-2 also appears to be on a subset of natural killer (NK) cells (Leu-2+, Leu-11+, Leu-4−, and cytolytic against K-562). In many transplant patients, these Leu-2+ NK cells predominate during lymphoid regeneration.[13] The presence of large numbers of Leu-2+ NK cells would tend to reduce the "helper/suppressor" ratio as measured by single parameter phenotyping. Therefore, the use of "helper/suppressor" T cell ratios may be misleading in this group of patients. Since his cells were not studied by multiparameter analysis, it is uncertain whether in this case the anti-Leu-2 labelled cells are NK cells or suppressor T cells. However, in this case there was clearly a reduction in the absolute number of Leu-3+ cells. These observations support the suggestion that the quantitation of subsets of T lymphocytes is a more appropriate indicator of HTLV-III infection than subset ratios.[1,5,19]

Although patients who undergo bone marrow transplantation are exposed to blood products from many donors, the risk of transmission of HTLV-III is small. It can be inferred from the recent reduction in the number of 17- to 35-year-old, male blood donors and HBsAg-positive blood donors[20–22] that publicity and FDA[23] guidelines have reduced the numbers of blood donors at high risk for transmitting HTLV-III. The recent general availability of anti-HTLV-III screening of all blood donors is expected to reduce this risk even further. However, due to the variable and long incubation period, some patients transplanted in the last one to five years may still be at risk. When patients without chronic GVHD develop opportunistic infections or lose immune reactivity more than one year after transplantation, consideration should be given to transfusion-associated AIDS.

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Natural Killer Cell Activity From Hemophiliacs Exhibits Differential Responses to Various Forms of Interferon

By David S. Matheson, Bridget J. Green, Man-Chiu Poon, Marvin J. Fritzler,
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Patients with hemophilia are at risk for the development of acquired immunodeficiency syndrome (AIDS). Patients with AIDS have recurrent infections and/or malignancy and altered immune response, including decreased T lymphocyte counts, decreased T helper lymphocytes, defective T cell blastogenesis, hypergammaglobulinemia, defective natural killer (NK) activity and impaired response of NK to interferon-β (IFN-β). It is feasible that chronic antigen stimulation with subsequent release of interferon could be related to the impaired NK reactivity to IFN-β of patients with AIDS. Because hemophiliacs are subjected to chronic antigen stimulation secondary to the administration of foreign protein, the reactivity of NK cells from patients with hemophilia to IFN-α, IFN-β and IFN-γ was studied. Eight patients with hemophilia requiring high levels of clotting factor replacement were assessed. Three patients were antibody positive to HTLV-III. All had normal baseline NK cell function. In the first set of experiments, all patients responded normally to in vitro IFN-α by increasing NK activity, but four patients had significant failure and two had mild impairment in NK response to IFN-β. This latter observation was particularly evident at very low concentrations of IFN. In repeated experiments, seven of eight had impaired NK response to IFN-β and IFN-γ but normal response to IFN-α. Only one patient's NK cells responded better to IFN-γ. There was no obvious correlation of these findings to antibody status to HTLV-III. Chronic antigen stimulation and the modulation of interferon receptors are discussed as possible mechanisms that could produce these findings.

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ACQUIRED immunodeficiency syndrome (AIDS) was first described in 1981 and is associated with opportunistic infections, malignancy, and abnormal cellular immune reactivity.[1] T cell leukemia virus type III (HTLV-III) is the putative etiologic agent.[2] Cellular immune abnormalities in AIDS include decreased number of helper T cells with decreased ratios of helper to suppressor T cells, decreased blastogenic response of T cells to mitogens and antigens, decreased natural killer (NK) cell level with an impaired response to interferon (IFN)-β and the presence of an acid labile IFN-α in the serum.[3-5] Patients with hemophilia are at risk for the development of AIDS,[6-8] possibly due to the transmissibility of an etiologic factor in blood products. The blood product associated with a higher risk for the development of AIDS in hemophiliacs is the lyophilized factor VIII product,[9,10] perhaps due to the large number of donor plasma units that are pooled to prepare this product. Several groups have reported cellular immune alterations in asymptomatic hemophiliacs.[9-15] Some groups found decreased helper to suppressor T cell ratio secondary to an increase in suppressor cells. Smolen et al demonstrated a decrease in autologous mixed lymphocyte response in hemophiliacs.[14] An increased frequency of activated T cells has been documented.[15]

The purpose of these investigations was to assess the responsiveness of NK cells from hemophiliacs to the various forms of interferon. Like patients with AIDS, it was found that a significant number of patients with hemophilia had a defective NK augmentation to IFN-β. They also had a decreased NK response to IFN-γ but were normally responsive to IFN-α. Unlike AIDS patients, the baseline level of NK was normal.

MATERIALS AND METHODS

Patients. Eight patients with hemophilia were studied. They were men ranging in age from 20 to 59 years. Patients 4 and 6 were factor IX deficient, and the remainder lacked factor VIII. All had procoagulant activity <0.01 U/mL. Replacement of factor during 1983 ranged from 21,000 U to 76,000 U, excluding one patient who required nearly 180,000 U for surgical procedures. Patient 7 had longstanding intermittent thrombocytopenia of unknown etiology. Patients 2, 5, and 7 were known to be antibody positive to HTLV-III. Only patient 3 had no significant titer to cytomegalovirus; all were hepatitis B antigen negative and all were antibody positive to Epstein-Barr virus viral capsid antigen.

Cells. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by centrifugation over Ficoll-Hypaque (Pharmacia, Montreal) from male controls or hemophiliac patients who had given informed consent. After they were washed, the cells were resuspended in RPMI-1640 (GIBCO, Burlington, Canada), in appropriate concentrations. Cell surface markers were performed by cytofluorographic analysis using the following reagents: OKT3, pan T cells; OKT4, helper T cells; OKT8, suppressor T cells; Leu 7, NK cells; and surface Ig, B cells.

Natural killer cell assay. The NK activity was assessed using a standard chromium release assay as published previously.[16] In brief, PBMCs were mixed with 10^4 K562 cells (Institute for Medical Research, Camden, NJ) which had been labeled with ^{51}Cr (Amersham, Montreal) to yield ratios of 30:1, 10:1, 3:1, and 1:1 effector to target cells; the cells were incubated for 16 hours. Percentage of lysis and lytic units per 10^7 cells were calculated according to standard techniques.[17] IFN-α and IFN-β were supplied in a partially purified form[18,19] by Dr Y. H. Tan, University of Calgary, Alberta, Canada. IFN-γ was purchased from Mulloy (Springfield, Va). Effects of IFN on the NK cell assay were assessed by preincubating 2×10^6 PBMC with varying amounts of IFN for two hours prior to aliquoting the cells into the microtiter plate for assessment of NK activity.

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Delayed hypersensitivity skin testing. The CMI-Multitest kit (Rhone-Poulenc, Montreal) was used.[20] The quantitative and qualitative responses were recorded at 48 hours as recommended by the supplier.

RESULTS

All patients had white blood cell counts above 3.0 × 10^9/L, and only patient 7 had lymphopenia of $<1 \times 10^9$ lymphocytes/L. Patient 7 also had a platelet count of 76 × 10^9/L; the platelet counts of the other patients were normal. Hypergammaglobulinemia was present in patients 2, 5, 6, and 8 in the IgG fraction. Patient 5 had an elevated IgA and patient 2 had an increase in his IgM level. The hypergammaglobulinemia was polyclonal in all cases as judged by protein electrophoresis. All patients had positive responses to at least one of the antigens tested by delayed hypersensitivity skin testing, using the CMI Multitest with a minimum recorded response of 3 mm of induration. Hence, none of the patients was completely anergic (data not shown).

Standard cell surface markers were assessed using monoclonal antibodies and a Becton Dickinson cytofluorograph (Table 1). All except patients 2 and 7 had an adequate percentage of T cells, and all except patients 5 and 7 had an adequate percentage of OKT4+ cells. Patient 5 had a decreased percentage of suppressor T cells. Patient 7 had decreased absolute helper and suppressor cell numbers. In patient 7, the helper (OKT4) to suppressor (OKT8) ratio was 0.6, for all others, it was >1.0. The percentage of Ig-positive B cells was normal and ranged from 5% to 11%. The percentage of Leu-7–positive cells ranged from 8% to 22% and was within normal limits. Hence, those patients with abnormalities in lymphocyte markers were those who had antibody to HTLV-III (patients 2, 5, and 7 from Table 1).

The baseline level of NK cell activity was adequate (Fig 1). Most patients did not respond well to IFN-β, but did respond to IFN-α. IFN-β at 100 U/2 × 10^6 PBMCs in 0.6 mL of medium failed to induce (or induced to a lesser extent) increments in the percentage of lysis when compared with IFN-α. The NK activity of PBMCs from hemophilia patients averaged 60.6% (± 11.2%) when incubated with IFN-α and 50.4% (± 7.3%) when treated with IFN-β. The baseline NK average was 48.0% (± 8.8). Using a paired *t* test, there was a significant difference between the IFN-α–induced and IFN-β–induced responses ($P < .001$). The NK response of PBMCs from controls run simultaneously with the hemophiliac PBMCs did not reveal any significant difference in response to IFN-α or IFN-β.

Table 1. Lymphocyte Markers and HTLV-III Antibody Status

Patient	OKT3	OKT4	OKT8	H/S Ratio	HTLV-III Antibody
1	74	29(574)	28(554)	1.1	–
2	41	21(222)	11(116)	1.9	+
3	56	25(380)	11(167)	2.2	–
4	53	27(518)	26(499)	1.0	–
5	68	11(335)	7(213)	1.6	+
6	54	28(541)	24(464)	1.2	–
7	28	12(100)	19(158)	0.6	+
8	56	34(571)	15(252)	2.3	–
Normal	50–70	20–46	10–35	>1.0	–

Marker data are expressed as percentage of peripheral blood mononuclear cells. Numbers in parentheses are WBC times percentage of lymphocytes times percentage of OKT reagent expressed as cells × 10^6/L.

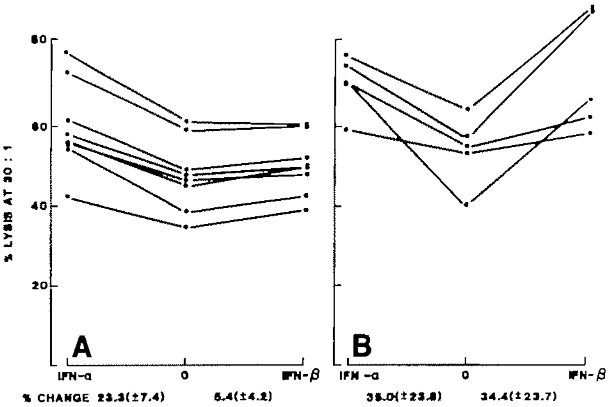

Fig 1. NK activity of the hemophilia patients (A) or controls (B) is shown at baseline prior to (center) and subsequent to exposure to interferon (IFN)-α (left) or IFN-β (right of each panel). IFN concentration was 100 U/2 × 10^6 peripheral blood mononuclear cells (PBMC) in 0.6 mL and natural killer (NK) activity is recorded at a 30:1 effector to target ratio. The average percentage of increase induced by each type of IFN [(NK with IFN–NK without IFN)/NK without IFN] times 100 is given below the figure.

To assess this phenomenon further, the response of the NK cells to low concentrations of IFN-α and IFN-β was assessed in four separate experiments (Fig 2). The control PBMCs demonstrated the same dose–response curves to IFN-α as to IFN-β. However, the PBMCs of the hemophilia patients 1, 5, 6, and 8 were markedly less responsive to IFN-β than to IFN-α. Patients 2 and 3 demonstrated the same pattern, but to a lesser degree; only patient 4 responded better to IFN-β. Patient 8 was assessed on two separate occasions; the results were reproducible in that each time the PBMCs responded to IFN-α but essentially did not respond at all to IFN-β. Hence, the NK response to IFN-β was significantly depressed in four of eight patients and mildly depressed in 2 others.

All patients were reassessed again in terms of their responsiveness to all three types of IFN, α, β and γ. From Fig 3, it can be seen that control PBMCs tend to respond similarly to IFN-α, IFN-β and IFN-γ. However, patients with hemophilia responded normally to IFN-α but poorly to IFN-β and IFN-γ (patients 2, 3, 4, 5, 6, and 8). Patient 1 responded better to IFN-γ and demonstrated no real difference in response to IFN-α and IFN-β. Patient 7 did not show any significant defect in response to the three forms of IFN.

Therefore, most patients with hemophilia demonstrated a defective NK response to IFN-β and IFN-γ but normal response to IFN-α. Although some exceptions in reproducibility exist (eg, patients 1 and 4 in Figs 2 and 3), most patients have a consistently impaired NK response to IFN-β (Figs 1, 2, and 3).

DISCUSSION

These data indicate that most patients had essentially normal routine hematology and marker data. Minor aberra-

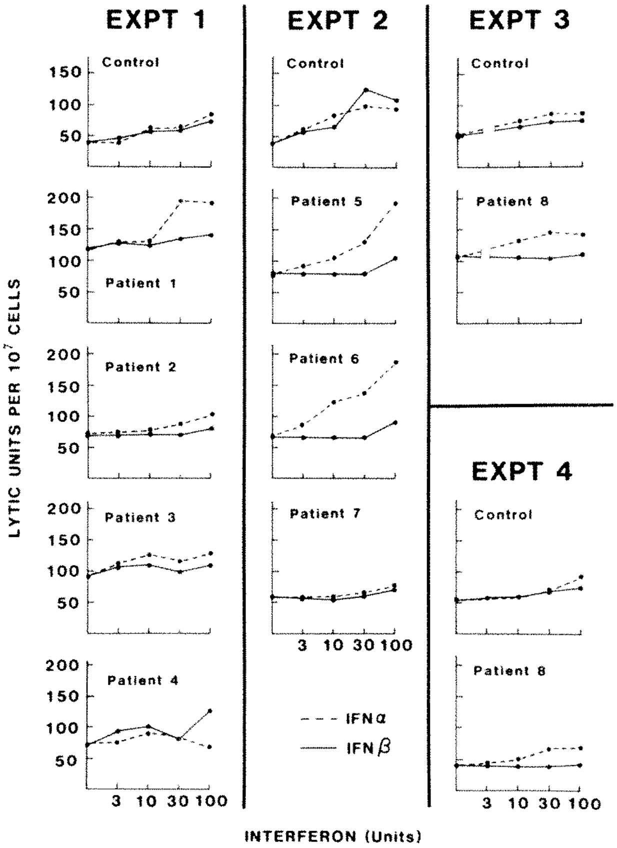

Fig 2. Four experiments comparing the natural killer (NK) response of PBMC from patients or controls to IFN-*α* (- - - -) or to IFN-*β* (——) are shown. IFN concentrations are expressed as U/2 × 10^6 peripheral blood mononuclear cells in 0.6 mL. NK activity is expressed as lytic units per 10^7 effector cells.

tions were present, especially in those who were antibody positive to HTLV-III. All patients were asymptomatic at the time of testing. The raised IgG or IgA in four of eight patients were polyclonal in nature. Taken together, these results may be more consistent with chronic antigen stimulation than with AIDS. However, since patients with AIDS have impaired NK response to IFN-*β*,[4] similar studies were performed with PBMCs from hemophiliacs.

The PBMCs from most patients with hemophilia failed to increase in NK level in response to IFN-*β* and IFN-*γ*. IFN-*β* is a glycoprotein secreted from fibroblasts during viral infections.[21] IFN-*α* is derived from white cells secondary to viral infection. IFN-*γ* is derived from blast-transformed lymphocytes. IFN-*β* and IFN-*γ* are glycoproteins in that they bind to Concanavalin-A (Con A) whereas IFN-*α* does not.[22,23] The receptor studies for IFN-*α*, -*β* and -*γ* (although not performed on NK cells) indicate that IFN-*β* can inhibit binding of IFN-*α* and IFN-*γ*, but that IFN-*α* and IFN-*γ* do not compete for the same binding sites.[24-27] This has been interpreted to mean that the receptor for IFN-*β* and IFN-*γ* may be carbohydrate dependent, but that the receptor for IFN-*α* and IFN-*β* is protein defined.[25] It is known that the presence of IFN in culture systems will decrease the number of IFN receptors.[28] The chronic exposure to IFN-*β* or IFN-*γ* could decrease this receptor density and impair responsiveness to IFN-*β*. Hence, it is feasible that there has been down modulation in the IFN-*β* and IFN-*γ* receptor. This is consistent with the argument presented for the diffuse hypergammaglobulinemia; that is, hemophiliacs are exposed to chronic antigen stimulation. The activated lymphocytes could then sustain increased IFN-*γ* levels and down regulate IFN-*γ* and IFN-*β* receptors. It has been shown that some patients with AIDS have an abnormal acid labile IFN-*α* present in their serum.[5] A similar acid-labile IFN-*α* has been demonstrated in 3 hemophiliacs prior to the onset of AIDS and was seen transiently in 3 of 46 asymptomatic hemophiliacs.[29] It is not

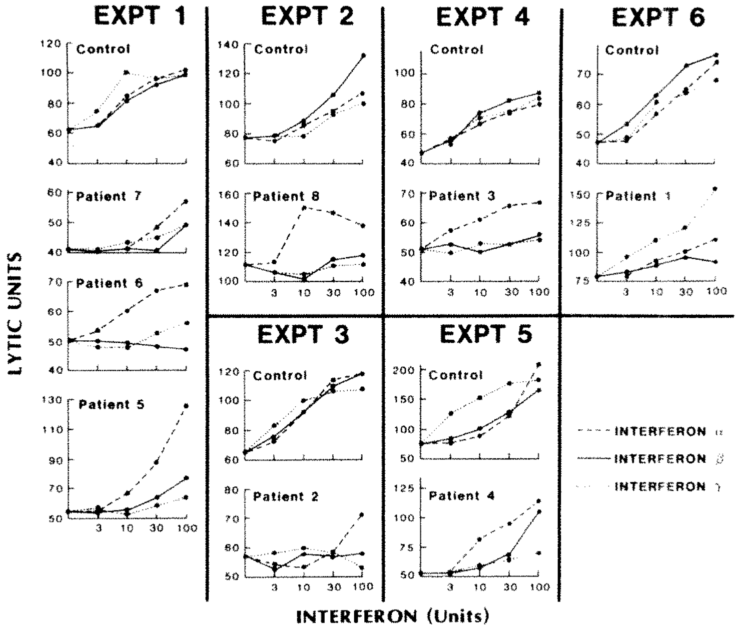

Fig 3. Six experiments comparing the natural killer (NK) response of peripheral blood mononuclear cells of patients and controls to IFN-*α* (- - - -), IFN-*β* (——), IFN-*γ* (· · · · ·) are shown. NK activity is expressed as in Fig 2.

known if this abnormal IFN modulates cellular reactivity through the IFN-α receptor.

Therefore, there is evidence for an impaired NK augmentation secondary to IFN-β and IFN-γ which was common in patients with hemophilia and which did not correlate with the presence of antibody to HTLV-III. These data are consistent with the suggestion that the receptors for IFN-β and IFN-γ are correlated, if not identical, and the receptor for IFN-α is different. Down modulation of IFN receptors may be secondary to chronic antigen stimulation, presumably as a result of the continuous requirement for foreign protein related to coagulant therapy.

ACKNOWLEDGMENT

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Expression of a Cloned Lepore Globin Gene

By Carl Dobkin, Jennifer Clyne, Aida Metzenberg, and Arthur Bank

Lepore globin is synthesized in markedly diminished amounts (~10% to 15% of normal β-globin) in human erythroid cells. To study the molecular mechanisms responsible for the diminished biosynthesis of Lepore globin, the Lepore–Boston gene was cloned from a charon phage DNA library and expressed in HeLa cells. Northern blotting and S1 nuclease analyses indicated that the Lepore gene produced less globin mRNA than a β-gene and more than a δ-gene. The results indicate that expression of the Lepore–Boston gene in HeLa cells is reduced to an extent comparable to that seen in erythroid precursors in vivo. This indicates that the decrease in Lepore globin gene transcription is due to the δ-nucleotide sequences either in the 5′ flanking region or within this gene.

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LEPORE GLOBINS are fusion products of δ- and β-polypeptide components.[1,2] Lepore–Boston consists of δ-globin sequence from its N terminal end to amino acid 87, and β-globin sequence from amino acid 116 to its C terminal end.[3] The crossover from δ-sequence to β-sequence in this Lepore gene has been studied by restriction analysis and has been shown to be near the 5′ end of the large intervening sequence (IVS 2), a region of strong homology between the δ- and β-genes.[4] Lepore globin is present in reduced amounts relative to normal β-globin levels in both heterozygotes and homozygotes. Lepore globin mRNA is present in reduced quantities as well, indicating that the decreased production of Lepore globin is due either to decreased transcription of the Lepore globin gene or to abnormal processing or instability of the Lepore globin mRNA.[5] To investigate this RNA reduction, we transferred a cloned Lepore globin gene into tissue culture cells and analyzed its expression. The results indicate that Lepore globin mRNA accumulates in HeLa cells transfected with this gene at a lesser rate than with a normal β-globin gene and at a greater rate than with the normal δ-globin gene. This is consistent with the relative expression of each of these genes in erythroid cells in vivo. The lack of aberrant splice products in the HeLa cell transcripts suggests that the decreased expression of the Lepore globin gene is due to decreased transcription or mRNA stability.

MATERIALS AND METHODS

A Lepore–Boston gene was cloned from a library of DNA fragments inserted into charon 27 and isolated by one of us (A.M.) from a typical patient (L.P.)[6] homozygous for hemoglobin Lepore–Boston.[4,5] The *Pst*I fragment containing the Lepore globin gene was subcloned into a plasmid, pBSV,[7] at the *Bam*HI site (Fig 1). To do this, we synthesized (on an Applied Biosystems Synthesizer) an eight-base oligomer, GATCTGCA, which is complementary to the *Pst*I and *Bam*HI overhanging ends. The ligation of this oligomer to the Lepore globin *Pst*I fragment[8] resulted in a fragment that could be inserted into the *Bam*HI site of pBSV (Fig 1). This plasmid contained approximately 0.5 kilobases (kb) of sequence 5′ to the structural Lepore globin gene and ~0.5 kb of 3′ to this gene. In these studies, δ-pBSV was also constructed from a *Pst*I fragment of an isolated δ-gene. The β-pBSV was also constructed, using a *Bgl*II fragment containing a normal β-globin gene that has ~1.5 kb of 5′ flanking sequence and 1.8 kb of 3′ flanking sequence.

HeLa cells were transfected with these plasmids using DEAE-Dextran.[9,10] RNA was isolated after 48 hours by cesium chloride sedimentation.[11] RNA was analyzed by Northern blotting,[12] by S1 nuclease protection,[10,13] and by RNAase protection when an RNA probe was used.[14] Three different probes were used in the analyses: a β-globin gene segment extending from the *Bal*I site 5′ to the gene to the *Eco*RI site in exon 3, in bacteriophage M13 mp 7, was used as template for synthesis of uniformly labeled probe.[13] A Lepore globin gene fragment extending from the 5′ *Pst*I site to the *Eco*RI site in coding region 3 was used in the same way. An RNA probe was prepared by inserting a *Bal*I–*Eco*RI fragment of the β-globin gene "downstream" from the SP6 promoter in the vector pSP62; uniformly labeled RNA was transcribed from this insert by SP6 RNA polymerase[14] using the instructions of the supplier (Promega Biotec or New England Nuclear, Boston). A 2.3-kb *Kpn*I–*Bam*HI SV 40 fragment was "nick translated" to probe Northern blots for T antigen transcripts.

Northern blots were prepared after agarose gel electrophoresis in the presence of formaldehyde[12] and were hybridized with β-globin probe. Uniformly labeled RNA probe at 0.3 Ci/mg was able to detect Lepore transcripts. S1 nuclease mapping was carried out using the Lepore and β-DNA probes.[10,13] A ^{32}P-labeled RNA probe was used in the analysis of globin transcripts in ribonuclease mapping experiments by hybridization of the cellular RNA with the probe and treatment of the hybrids with ribonucleases A and T1.[14]

RESULTS

RNAs transcribed in HeLa cells from the Lepore, β-, and δ-pBSV constructs were compared. A "nick-translated" β-globin probe was used in initial Northern blotting experiments, which showed a strong signal from the β-pBSV transcripts, but no signal in RNA from HeLa cells transformed with either the Lepore plasmids or the δ-plasmids (data not shown). This implied that β-globin mRNA accumulates in much higher quantities than δ- and Lepore RNAs. To improve the detection of Lepore RNAs and δ-RNAs, a uniformly labeled RNA probe representing the *Bal*I–*Eco*RI globin region was synthesized with bacteriophage SP6 RNA polymerase.[14] In this experiment, the β-globin RNA signal could easily be seen, and a weaker but detectable signal was also seen with the Lepore globin gene in the 10S RNA region (Fig 2A). There was no visible

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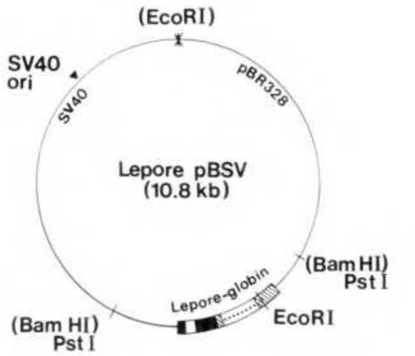

Fig 1. Lepore pBSV expression plasmid. The *Pst*I fragment of the cloned Lepore gene was ligated to a synthetic oligomer, GATCTGCA, in order to create a *Bam*HI end, and inserted into the *Bam*HI site of pBSV. The *Eco*RI site of pBSV had been eliminated. The *Pst*I fragment contains approximately 0.5 kb 5′ and 0.5 kb 3′ to the gene, which is shown cap site to poly A site, left to right. Open rectangles indicate IVS 1 (δ-sequence). Open rectangle with dots indicates IVS 2 (β-sequence). Solid rectangles indicate exon I and part of exon II, which are δ-sequence. Hatched rectangles indicate the remainder of exon II and exon III, which are β-sequence.

δ-globin signal, however. To provide an internal control for plasmid transfection and RNA recovery, RNA from cells transfected with β- and Lepore globin gene containing plasmids were assayed for the presence of transcripts from the plasmid T antigen gene. RNA from cells transfected with either Lepore globin plasmids or β-globin plasmids contains similar amounts of SV 40 T antigen RNA (Fig 2B). Thus, the decrease in Lepore globin mRNA as compared with β-globin mRNA in these same RNA samples is not due to differences in transfection or RNA isolation, but to differences in expression of the β-globin genes and the Lepore globin genes. These results indicate that the Lepore globin gene is expressed in HeLa cells at a level less than that of the β-globin gene and greater than that of the δ-globin gene, which is consistent with the expression of these genes in erythroid cells in vivo.

To further investigate the transcription and processing of RNA from these cloned genes, we analyzed them by S1 nuclease mapping. Using a Lepore DNA probe, a signal was seen with RNAs from HeLa cells transfected with β and Lepore genes but not consistently from those with the δ gene. The Lepore segments protected by Lepore RNA are, as expected, only the 145, 225 and 49 classes representing exons I, II, and part of III (Fig 3). The β-globin gene transcripts protected the same 145, 225, and 49 classes, as well as the 160 and 65 sizes, representing further cleavage of the 225 exon II fragment. The signal is much weaker with RNA from cells transfected with the δ-globin gene. No unexpected fragments were protected by the β-transcripts, the Lepore transcripts, or the δ-transcripts, indicating the absence of aberrant splicing. When a β-globin DNA probe was used, the probe segments protected by β-globin RNAs and Lepore globin RNAs were consistent with the predicted homology to the probe; β-globin RNA protects segments of 145, 225, and 49 bases, representing exons I, II, and part of III (Fig 4A). Lepore RNA signal was weaker than β-globin RNA signal even though twice as much Lepore RNA was used.

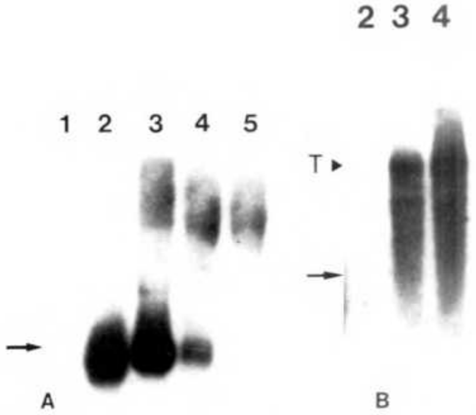

Fig 2. (A) Northern blot analysis of Lepore gene transcripts. Total RNA from HeLa cells transfected with β-, Lepore, or δ-globin gene plasmids was fractionated on 1% agarose gels in the presence of formaldehyde, transferred to nitrocellulose membrane, and hybridized with uniformly labeled RNA representing part of the β-globin gene. Lane 1: 25 μg HeLa RNA (no plasmid). Lane 2: 0.020 μg RNA from human reticulocytes containing approximately 1% β-globin mRNA. Lane 3: 25 μg RNA from HeLa cells transfected with β-globin plasmid. Lane 4: 25 μg RNA from HeLa cells transfected with Lepore plasmid. Lane 5: 25 μg RNA from HeLa cells transfected with δ-globin plasmid. Arrow at left points to the region that contains normal β-globin transcript (10S). (B) Northern blot analysis of "control" T antigen transcripts. Samples of the RNA analyzed in (A) were fractionated and transferred as in (A) and hybridized with nick-translated DNA containing the SV 40 T antigen region. Lane 2: 0.02 μg reticulocyte RNA. Lane 3: 25 μg RNA from HeLa cells transfected with β-globin plasmid. Lane 4: 30 μg RNA from HeLa cells transfected with Lepore plasmid. All the transfected plasmids contain the T antigen gene, and its transcription should be independent of the globin gene insert. The T antigen signal (T in margin) indicates that the transfection of the β-globin and Lepore plasmids was equivalent, as was the recovery of the transcribed RNA. This blot was rehybridized with a β-globin probe (as in [A]) (data not shown); the β-globin lane gave a strong 10S signal (at arrow), while only a trace of hybridization was detected in the Lepore lane.

When an RNA probe identical to the β-globin DNA probe was hybridized to β-, δ- and Lepore RNAs, and the hybrids are digested with RNAase A and T1, essentially the same pattern of signals is seen as with the DNA probe (Fig 4B). Again, the signal was greatest with β-RNA and substantially reduced with Lepore RNA. The signal with Lepore RNA was again greater than with the δ-RNA.

DISCUSSION

A cloned Lepore–Boston globin gene has been expressed in HeLa cells, and its expression compared to cloned normal δ- and β-human globin genes in this system. The results indicate that the Lepore globin gene is intermediate in its expression between that of normal β- and normal δ-genes, similar to the situation that occurs in vivo in human bone marrow. This suggests that the expression of these transfected genes is regulated by sequences contained within the fragments of DNA transfected. The Lepore gene differs from the β-gene by having δ-sequences in the 5′ flanking region as well as in exon I, the small intervening sequence (IVS 1), and exon II. The decreased expression of the Lepore

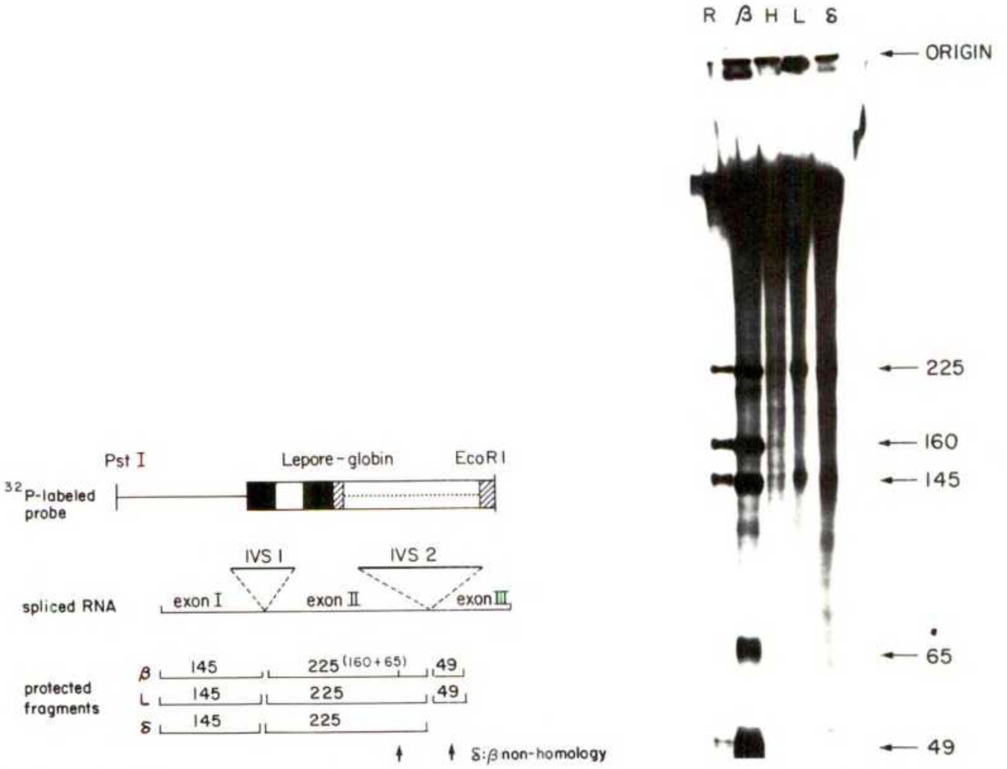

Fig 3. S1 mapping of HeLa cell transcripts with Lepore probe. RNA from HeLa cells transfected with β-, Lepore, or δ-globin gene plasmids was hybridized to uniformly lableled DNA, the hybrids were digested with S1 nuclease, the probe fragments were separated on 5% polyacrylamide (19:1, acrylamide:bis acrylamide; 7 mol/L urea) and visualized by autoradiography. The probe was synthesized on a single-stranded template representing the *Pst*I-*Eco*RI segment of the Lepore gene and isolated after *Pst*I and *Eco*RI digestion. In the scheme at bottom, the probe is represented as nontranscribed region (line); exon I and part of exon II, which are δ-sequence (solid rectangles); IVS 1, which is δ-sequence (open rectangle); part of exon II and exon III, which are β-sequence (hatched rectangle); and IVS 2, which is β-sequence (open rectangle with dots). The derivation of probe fragments protected by the different RNAs is also diagramed at bottom. Fragments protected by β-RNA are 145 bases (exon I), 225 bases (exon II), and 49 bases (part of exon III); nonhomology between probe and transcript (arrows at bottom) leads to some S1 cleavage of exon II fragment into 160- and 65-base fragments. (See lanes R and β.) Fragments protected by Lepore RNA are 145 bases (exon I), 225 bases (exon II), and 49 bases (part of exon III). (See lane L.) Fragments protected by δ-RNA are 145 bases (exon I) and 225 bases (exon II); nonhomology in exon III between probe and δ-transcript prevents protection in this region. (See lane δ.) Lane R: protection by 0.020 μg RNA from human reticulocytes (65-base signal is very light). Lane β: protection by 50 μg RNA from HeLa cells transfected with β-globin plasmid. Lane H: protection by 50 μg RNA from HeLa cells (no plasmid). Lane L: protection by 100 μg RNA from HeLa cells transfected with Lepore plasmid (49-base signal is very light). Lane δ: protection by 100 μg RNA from HeLa cells transfected with δ-globin plasmid.

gene as compared with the normal β-globin gene is probably due to these δ-sequences.

A gene constructed in vitro that resembles the Lepore gene also gives markedly decreased expression when transfected into a line of monkey kidney cells.[15] (In these experiments, the *Bam*HI 1.8-kb fragment of the δ-gene was ligated to a 3′ β-fragment at the common *Bam* site within these genes.) Recent experiments in our laboratory[16] indicate that the expression of a β-globin gene in HeLa cells is not decreased by substitution of a δ-IVS 1 for a β-IVS 1. Because exons I and II of these genes are >90% homologous, these results suggest that the δ 5′ flanking sequences of the Lepore gene are responsible for its decreased expression compared with the normal β-globin gene. Other experiments in our laboratory indicate that substitution of δ-IVS 2 for β-IVS 2 leads to markedly reduced expression. Thus, δ-IVS 2 either decreases the expression of a β-globin gene or β-IVS 2 is required for the high-level expression of this gene. The presence of β-IVS 2 in the Lepore globin gene may explain why it is expressed at a higher level than the δ-globin gene. Thus, elements within as well as flanking the δ- and β-globin genes seem to control their activity—the 5′ flanking region and IVS 2. The Lepore gene with a 5′ δ-flanking region, but a normal β-IVS 2, has a higher rate of expression than the normal δ-gene. These experiments do not directly demonstrate the precise step in RNA synthesis responsible for decreased Lepore mRNA accumulation. In addition, further experiments are required to determine whether the 5′ flanking regions and IVS of the δ-, β-, and Lepore genes have these effects when the cloned genes are expressed in erythroid cells.

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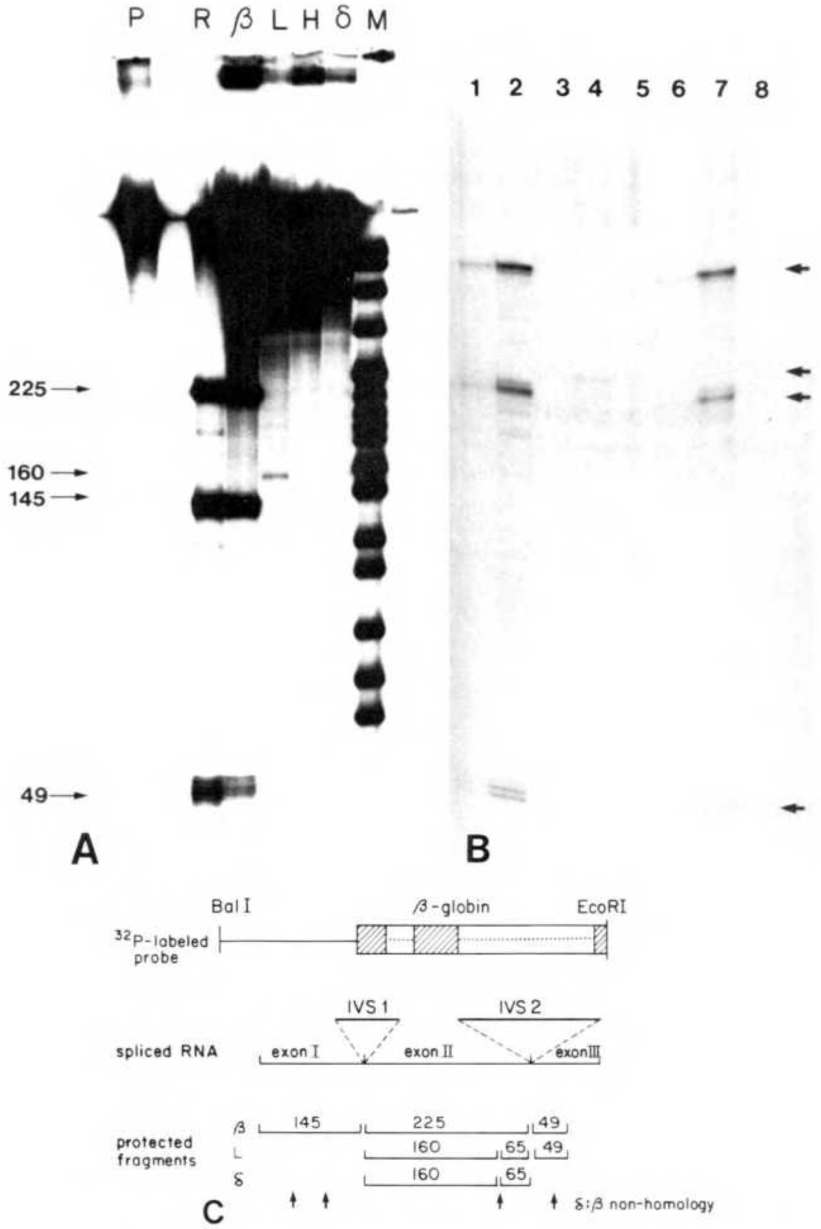

Fig 4. (A) S1 mapping of HeLa cell transcripts with β-globin DNA probe. RNA from HeLa cells transfected with β-, Lepore, and δ-globin gene plasmids was hybridized to uniformly labeled DNA, the hybrids were digested with S1 nuclease, the probe fragments were separated on 5% polyacrylamide (19:1, acrylamide:bis-acrylamide; 7 mol/L urea) and visualized by autoradiography. The probe was synthesized on a single-stranded template representing the *Bal*I–*Eco*RI segment of β-globin DNA and isolated after restriction enzyme digestion. In (C) the probe is represented as nontranscribed region (line), exons I, II, and part of III (hatched rectangles), and IVS 1 and 2 (open rectangles with dots). The derivation of probe fragments protected by the different RNAs is also diagramed at bottom. Fragments protected by β-RNA are 145 bases (exon I), 225 bases (exon II), and 49 bases (part of exon III). (See R and β.) Fragments protected by Lepore RNA are 160, 65 (representing parts of the 225-base exon II segment which is cleaved by S1 due to nonhomology between probe and transcript), and 49 bases (part of exon III). (See L.) Fragments protected by δ-transcripts are 160 and 65 bases (representing exon II), and the same fragments are seen with Lepore transcripts. (See lane δ.) Lane P: undigested probe. Lane R: protection by 0.020 μg RNA from human reticulocytes. Lane β: protection by 50 μg RNA from HeLa cells transfected with β-globin plasmid. Lane L: protection by 100 μg RNA from HeLa cells transfected with Lepore plasmid. Lane H: protection by 50 μg RNA from HeLa cells (no plasmid). Lane δ: protection by 100 μg RNA from HeLa cells transfected with δ-globin plasmid. (The 160-base fragment of exon II is barely discernible.) Lane M: *Hpa*II digested pBR 322 end-labeled marker. (B) Ribonuclease mapping of HeLa cell transcripts with RNA probe representing the β-globin gene. RNA from HeLa cells transfected with β-, Lepore, or δ-globin gene plasmids was hybridized to uniformly labeled RNA, the hybrids were digested with ribonucleases A and T1, the probe fragments were separated on 8% polyacrylamide (19:1, acrylamide:bis acrylamide; 7 mol/L urea) and visualized by autoradiography. The probe was synthesized on a *Bal*I-*Eco*RI β-globin fragment that had been subcloned (with an *Eco*RI linker) into the *Eco*RI site "downstream" from the SP6 promoter in pSP62. Plasmid DNA linearized with *Aat*I (New England Biolabs) was used as template for in vitro synthesis with SP6 RNA polymerase (New England Nuclear or Promega Biotec).[11] This probe is essentially the RNA analog of the probe described in (A). The derivation of the probe fragments is also similar, but additional fragments are visible in Lepore (lane 4); nonhomology between probe and transcript yields more discrete fragments than in the S1 mapping with a DNA probe. Arrows at right locate approximately (from top to bottom) 225-, 160-, 145-, and 49-base fragments. Lanes 1 and 2: protection by 0.020 and 0.050 μg RNA from human reticulocytes. Lane 3: protection by 50 μg HeLa RNA (no plasmid). Lane 4: protection by 100 μg RNA from HeLa cells transfected with Lepore plasmid. Lane 5: protection by 100 μg RNA from HeLa cells transfected with δ-globin plasmid. Lanes 6 and 7: protection by 10 and 25 μg RNA from HeLa cells transfected with β-globin plasmid. Lane 8: *Hpa*II digested pBR 322 end labeled marker. In lane 4, the Lepore RNA lane, a discrete 160-base band is seen as expected (see legend to [A]).

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Plasma-Mediated Alterations of Erythrocyte Deformability by Perfluorochemical Blood Substitutes

By Gretchen M. Holloway, E.A. O'Rear, and B.M. Fung

The effect of perfluorochemical blood substitutes (eg, Oxypherol or Fluosol-DA) on red cell deformability was investigated because these emulsions are in direct contact with red cells when they are used as temporary circulatory aids. Erythrocyte deformability was assessed by a constant volumetric flow rate filtration method. The results of in vitro incubation experiments indicate that perfluorotributylamine causes the deformability of human red cells to decrease significantly in the presence of plasma. However, there is no obvious loss in the deformability when washed cells are used. Neither mean cell volume nor white cells appear to be responsible for the observed effects of perfluorotributylamine. Perfluorodecalin and perfluorotripropylamine, two perfluorochemical compounds that are widely applied clinically, do not induce significant changes in red cell deformability with or without plasma. These results indicate the need for in vitro testing in the development of perfluorochemicals as blood substitutes.

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PERFLUOROCHEMICAL (PFC) blood substitutes are being actively developed and tested in many countries. They have the potential to provide a temporary circulatory aid, which could alleviate many current problems with transfusions, namely, availability and storage of blood, matching of blood types, and disease transmission. In addition, PFC suspensions show promise in several medical procedures, including the perfusion of donor organs,[1] the treatment of ischemia,[2-4] the improvement of coronary angioplasty,[5] and the enhancement of radiation therapy of tumors.[6]

Development of these red cell substitutes has not been without complications. For example, vapor emboli and prolonged retention times were observed in animal studies of early candidate PFCs. More recently, clinical trials in the United States have indicated increased pulmonary artery pressures, bradycardia, and chest pain[7] and complement activation[8] in some patients after the infusion of Fluosol-DA. With intimate contact between the PFCs and blood components, we anticipated that problems could arise if erythrocyte deformability were affected.

The determining factor of the life span for a typical red cell appears to be the deformability of that cell.[9] As the cell stiffens with age, it becomes more susceptible to splenic sequestration and removal from the circulation. Abnormal red cell flow properties, as measured by a variety of techniques, have been implicated as one mechanism of cell loss in a number of diseases and clinical conditions.[10,11] As a factor in certain types of anemia, reduced deformability translates to an accelerated rate of removal of cells by the microcirculation.

As a consequence of abbreviated red cell life spans, altered flow properties in the presence of a PFC could cause decreased tissue perfusion. Hence, the effect of PFC emulsions on erythrocyte deformability is an appropriate concern in their development as blood substitutes. The potential significance of this problem is augmented by the short half-lives for the current PFC emulsions,[12,13] so that the responsibility for oxygen transport requirements must be rapidly reassumed by the patient's own erythrocytes.

To date, no one has investigated erythrocyte deformability after exposure to PFC suspensions, even though the results of related experiments present reason for concern. In mongrel dogs, for instance, increased postmortem liver and spleen weights and larger-than-normal red blood cell volumes have been associated with the use of PFC suspensions.[14] In baboons infused with a PFC emulsion, hematocrit continued to decline beyond the hemodilution level after infusion.[15] Moreover, direct observation of red blood cells in vivo within the microvasculature of rabbit omentum has shown crenated red cells and erythrocytic debris on infusion of emulsions of perfluorotributylamine.[16,17] Using a filtration method, we have studied the effect of several PFCs on the deformability of erythrocytes. The PFC systems studied were Oxypherol, Fluosol-DA, and other emulsions prepared in this laboratory. Compositions of these systems are described later in detail.

MATERIALS AND METHODS

Erythrocytes were obtained from fresh human blood donated by healthy, nonsmoking male volunteers from whom prior informed consent had been obtained. Whole blood was used directly for some incubations, but in each case, red cells were resuspended in Ringer's/human albumin before the deformability test. Erythrocyte samples were washed twice each with normal saline and then Ringer's solution. Washing involved centrifugation, removal of supernatant, and resuspension of the cells in order to remove plasma and most of the white blood cells. Finally, the washed cells were resuspended in a solution appropriate for the ensuing procedure.

Erythrocyte deformability was measured using a constant volumetric flow rate filtration technique, in which pressure is used as an indication of the flow resistance through the 3-μm pores of a Nuclepore filter. The pressure *v* filtration time curve becomes linear with a slight positive slope after a short transient period; an initial pressure drop (P_0) is obtained by extrapolation of the linear region back to zero time when plugging by contaminant white cells or thrombi does not occur. Tests in this study were at a volumetric flow rate of 2 mL/min and hematocrit of 3% to 4% in Ringer's/human albumin. Details of the filtration method have been described previously.[18,19] Cell counts for each sample were adjusted with a Boehringer-Mannheim HC-333 cell counter (Houston); the hemato-

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crit was determined in duplicate by the microhematocrit method. Cell morphology was checked by light microscopy of wet mounts.

In order to investigate the mode by which the PFCs may affect the deformability of erythrocytes, the blood samples were treated in several ways:

1. The erythrocytes were harvested immediately after the blood was drawn, resuspended in a 0.05 g/L Ringer's albumin (human) solution, and then incubated with Oxypherol.
2. Whole blood was incubated with Oxypherol, or another PFC emulsion to be tested, before the erythrocytes were separated.
3. White cells, for the most part, were removed from the whole blood; the recombined plasma and erythrocyte suspension was subsequently incubated with Oxypherol.
4. The plasma was first incubated at 56 °C for one hour to denature the proteins; then it was centrifuged to remove the flocculated proteins; finally, the cells were resuspended in the treated plasma and mixed with Oxypherol before incubation.

Samples were washed and the PFCs removed before the filtration tests. Oxypherol and Fluosol-DA emulsions and annex solutions were obtained from Alpha Therapeutics Corporation and prepared according to instructions. Except where noted, incubations were at 37 °C for four hours; the total time for workup, incubation, and measurements was less than eight hours in all experiments. PFC emulsions were added to 10 vol%, roughly equivalent to the transfusion of 1 unit. In each case, a control was run with cells prepared in a similar manner but without the PFC emulsion.

RESULTS AND DISCUSSION

When Oxypherol was added to washed erythrocytes, with incubation, it did not affect the deformability of the erythrocytes (Table 1, part A). However, when Oxypherol was first incubated with whole blood (Table 1, part B) or plasma (Table 1, part C), it caused the erythrocytic deformability to decrease significantly (Gosset's *t* test). To confirm that the flow properties had been altered, viscosities of red cell suspensions (70% hematocrit) obtained from paired whole blood samples incubated four hours with and without Oxypherol were measured in a capillary tube viscometer (1.15-mm ID). At wall stress $\tau_w = 2.99$ dyn/cm^2, suspension viscosity increased after incubation with Oxypherol (controls, 13.5 ± 2.6 cp (5); Oxypherol, 16.2 ± 4.4 cp (5); $P < .15$). A high hematocrit was used in these tests because, according to Dintenfass,[20] red cell "rigidity" becomes a significant factor for viscosity only at higher cell concentrations. The mean cell volume (MCV) was calculated, and unlike previous results for canine cells, no changes due to experimental procedures were observed (Table 2), although the MCV varied slightly among individuals. Results for MCV compared favorably with reference values for normal cells.[21,22] Resuspended in Ringer's/albumin, red cells for all samples exhibited normal morphology. The deformability tests indicate that one or more of the components in the plasma causes the Oxypherol to interact with the erythrocytes. When the plasma was first incubated at 56 °C for one hour (and the denatured proteins were removed before being incubated with the red cells and Oxypherol), the effect of the PFC emulsion on the erythrocyte deformability was reduced (Table 1, part D). However, there was also an apparent increase in the erythrocytic deformability (ie, the pressure drop decreased) of the denatured-plasma control compared with the whole blood control. As a result, a difference between the control and the sample treated with Oxypherol was still observed in this case; but unlike samples incubated with undenatured plasma, this difference was not statistically significant. Therefore, we speculate that the decrease in the red cell deformability may be due to plasma protein-mediated transfer of the PFC component in Oxypherol to the membrane of the erythrocyte. The validity of this hypothesis and the component responsible for the transport of the PFC remains to be tested. Alternatively, the particular mechanisms of reduced deformability might be enhanced platelet activation[23] or protein adsorption. The former of these two is particularly suspect, since a recent report indicates that platelet activation can affect red cell deformability.[24] Additional studies are planned to investigate these mechanisms. Because results for recombined plasma with Oxypherol were similar to those for whole blood with Oxypherol, it appears that white blood cells are not responsible for the observed changes (Table 1, parts B and C).

Table 1. Results of Erythrocyte Deformability Measurements

Samples	Composition	Initial Pressure Drop (mm Hg) ± SD	Statistically Significant Difference ($P < .01$)
A. Prewashed cells	Control	21.2 ± 1.5 (5)	—
	Oxypherol	21.8 ± 2.3 (5)	No
B. Whole blood	Control	19.2 ± 2.7 (12)	—
	Oxypherol	26.0 ± 3.8 (12)	Yes
	Oxypherol ± PC	27.0 ± 2.9 (4)	Yes
	PFTBA ± PFD	28.4 ± 1.9 (4)	Yes
	Fluosol-DA	21.9 ± 0.2 (3)	No
	Pluronic F-68	18.5 ± 1.2 (4)	No
C. Plasma	Control	19.3 ± 3.4 (6)	—
	Oxypherol	24.9 ± 1.3 (6)	Yes
D. Denatured plasma	Control	14.8 ± 5.1 (5)	—
	Oxypherol	18.6 ± 8.8 (5)	No

A larger pressure drop of a sample compared with the control indicates a loss in deformability. The last column shows whether or not the difference between the sample and the corresponding control is statistically significant within a 99% confidence limit. PC, phosphatidylcholine; PFTBA, perfluorotributylamine; PFD, perfluorodecalin.

Table 2. Mean Cell Volume After Incubation for Four Hours at 37 °C

Samples	Composition	MCV (μm^3) ± SD	Statistically Significant Difference ($P < .01$)
Prewashed cells	Control	81.7 ± 11.4 (3)	—
	Oxypherol	88.8 ± 6.2 (3)	No
Whole blood	Control	88.9 ± 4.8 (11)	—
	Oxypherol	92.2 ± 6.4 (10)	No
	Oxypherol + PC	89.9 ± 3.0 (4)	No
	PFTBA + PFD	89.4 ± 1.2 (4)	No
	Fluosol-DA	90.1 ± 4.2 (3)	No
	Pluronic F-68	88.4 ± 2.8 (4)	No
Plasma	Control	84.3 ± 5.4 (6)	—
	Oxypherol	87.1 ± 8.7 (6)	No
Denatured plasma	Control	85.6 ± 3.8 (5)	—
	Oxypherol	90.0 ± 3.6 (5)	No

Previous workers have related toxicity of some PFC emulsions to particle size. Electron microscopy results show that, when prepared by established methods, the average particle diameter of perfluorotributylamine (PFTBA)-Pluronic F-68 emulsions corresponds to a safe value for infusion.[25] Similarly, the mean particle size for perfluorodecalin–perfluorotripropylamine (PFD–PFTPA) and Pluronic F-68 emulsions is reported to be 0.1 μm.[26,27] According to literature supplied by Alpha Therapeutics, Oxypherol and Fluosol-DA emulsions have an average particle size of 0.1 to 0.2 μm. Using a centrifugation method,[28] we obtained mean particle sizes of less than 0.12 μm for Fluosol-DA and of 0.25 μm for Oxypherol.

Because we found that the Oxypherol decreases the deformability of erythrocytes, it is important to examine whether or not other PFC blood substitutes have a similar adverse effect. The PFC in Oxypherol is PFTBA (20.0%) and the emulsifier is Pluronic-F68 (2.56%). Because of the long retention time of PFTBA in the liver and spleen, it is generally used for animal tests but not for clinical purposes. The PFC preparation that has been tested extensively in Japan and China and to a lesser degree in the United States is Fluosol-DA, 20%. Its PFC components are 14% PFD and 6% PFTPA, while 0.4% yolk phosphatidylcholine (PC) and 0.032% potassium oleate are used as co-emulsifiers in addition to 2.7% Pluronic-F68. The other components (3.0% ethyl starch as an oncotic agent, 0.60% NaCl, 0.034% KCl, 0.020% $MgCl_2$, 0.08% $CaCl_2$, and 0.21% $NaHCO_3$ as osmotic and buffering agents, and 0.18% glucose) are identical in the two types of emulsions. Fluosol-DA also has 0.80% glycerol for viscosity control, but this is not likely to affect the results as discussed below.

When Fluosol-DA was used to replace Oxypherol, it did not show a statistically significant alteration in erythrocytic deformability (Table 1, part B); in light of the findings for Oxypherol, this was an important negative result. This could be due to the presence of PC or a difference in the PFCs. To examine whether or not PC protects erythrocytes against the action of Oxypherol, we added the phospholipid to Oxypherol, sonicated the system to ensure homogeneity, and tested the emulsion in the incubation system. The results were not different from those of Oxypherol alone (Table 1, part B). Therefore, the decrease of the erythrocyte deformability must be due to different properties of the different PFCs. To ascertain this point, we made another emulsion that had the same composition as Fluosol-DA, except that PFTPA was replaced by PFTBA. This emulsion decreased the deformability of erythrocytes in the same way as Oxypherol (Table 1, part B). Finally, the emulsifier Pluronic F-68 alone was tested to make sure that it was not the cause of the decrease in erythrocytic deformability. No adverse effect was found (Table 1, part B). This negative result was not a consequence of different batches of Pluronic F-68 because the same material was ued in the PFTBA + PFD experiments. Moreover, the mean particle sizes above indicate that Oxypherol has less interfacial area available to the surface agent Pluronic than does Fluosol-DA. If mass transport of Pluronic were important in affecting the erythrocytic deformability, a smaller surface area would have less effect. This is contrary to what was observed.

From the results shown in Table 1 and the above discussion, we conclude that PFTBA causes the deformability of human erythrocytes to decrease considerably in the presence of plasma, whereas PFTPA and PFD have comparatively little effect. Many techniques have been used to assess erythrocyte deformability, although the sensitivity of various testing methods relative to the physiological deformation requirements has not been clearly established. However, at least one clinical trial with Fluosol-DA is consistent with observations of this study.[12,13]

The results of these experiments have an important implication beyond the loss of patient red cell mass because it may be related to other biological effects of the PFCs, such as their different retention times in the body and different cytotoxicities. It has recently been reported that a 25% emulsion of PFTBA is selectively cytotoxic to macrophages,[29] but it is not clear whether PFTPA and PFD have a similar effect. It is of interest to note that the effect of Oxypherol (an emulsion of PFTBA) on neutrophils[30] is much larger than that of Fluosol-DA (which contains PFTPA and PFD). Through the use of nuclear magnetic resonance, electron microscopy, and differential scanning calorimetry, we have also found that the physical state of PFTBA in phospholipid bilayers is quite different from those of the other two PFCs. Fortunately, PFTBA preparations are not used for clinical purposes. We suggest that in vitro tests, such as cytotoxicity, erythrocytic deformability, and blood compatibility, of these and other PFCs continue to be important to their development in clinical applications and should be made in parallel with animal tests. The adverse effect of one PFC does not necessarily mean that other PFCs would display similar effects, but it does emphasize the need for further testing to find the most suitable candidates for use as blood substitutes.

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Donor-Derived Red Blood Cell Antibodies and Immune Hemolysis After Allogeneic Bone Marrow Transplantation

By Jill Hows, Keith Beddow, Edward Gordon-Smith, Donald R. Branch, Wayne Spruce, Irena Sniecinski, Robert A. Krance, and Lawrence D. Petz

Six cases of immune hemolytic anemia attributed to *donor*-derived red cell antibodies after allogeneic bone marrow transplantation (BMT) are reported. In 2/6 cases, severe intravascular hemolysis was seen, 6/6 required increased red cell transfusion, and 1/6 was treated by plasma exchange. All recipients were receiving cyclosporine to prevent graft-*v*-host disease. Investigations showed that in each case, the donor lacked ABO or Rho(D) red cell antigens present in the recipient. The direct antiglobulin test was positive in 6/6. Relevant serum antibody (anti-A, four cases; anti-B, one case; anti-D, one case) was first detected one to three weeks after BMT. Eluates made from recipient red cells showed the same specificity as serum antibody. Maximum hemolysis occurred nine to 16 days after BMT, suggesting that active production of antibody by "passenger" donor lymphocytes was the likely mechanism of hemolysis, rather than passive transfer of antibody in the marrow infusion. Retrospective analysis of 21 consecutive cyclosporine-treated BMT patients receiving marrow lacking ABO or D antigens present in the recipient showed that (1) 15/18 patients tested had red cell antibody production against recipient red cell antigens; (2) despite the frequent presence of antibody specific for recipient red cell antigens, only 3/21 patients developed clinically significant hemolysis; (3) clinical hemolysis could not be predicted by donor or recipient red cell antibody titers. We conclude that although red cell antibody against recipient antigens is frequently produced after minor ABO and D mismatched BMT in cyclosporine-treated recipients, only 10% to 15% of cases develop clinically significant immune hemolysis. The data presented show that the most likely source of antibody is "passenger" donor lymphoid cells.

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ALLOGENEIC bone marrow transplantation (BMT) in humans involves the transfer of mature donor lymphocytes to the recipient. It is theoretically possible for transferred donor lymphocytes to produce antibodies specific for recipient red cell antigens that are lacking on the donor's own red cells. If this occurs, it could present clinically as immune hemolysis in the posttransplant period. Conceivably, complications such as renal failure could occur in association with severe hemolysis.

We report six bone marrow transplantation (BMT) cases in which the appearance of red cell antibodies was associated with clinical and laboratory evidence of immune hemolysis. The peak time for hemolysis was one to two weeks after BMT, making passive transfer of plasma antibody in the marrow infusion an unlikely cause. From the data presented, we suggest that active production of antibody by donor lymphocytes stimulated by recipient RBC antigens is the cause of the hemolysis. To determine the frequency of hemolysis due to this mechanism, we have retrospectively analyzed 97 consecutive cyclosporine-treated BMT patients from one center (Hammersmith). The association between cyclosporine and this phenomenon was strengthened by our failure to find, on retrospective analysis, a single case of immune hemolysis or donor-derived antibody production in 13 patients treated with methotrexate in the postgraft period.

MATERIALS AND METHODS

Patients

Group 1. Six patients from three transplant centers who presented clinically with immune hemolysis after BMT were investigated. Informed consent to investigations was obtained according to each of the transplant center's ethical committee guidelines. Clinical and laboratory data are shown in Table 1. Hematologic and biochemical data illustrating a typical case are shown in Fig 1. Patients 1 through 5 were transplanted from HLA-identical sibling donors and patient 6, from an HLA phenotypically matched unrelated donor. In cases 1 through 5, the donors lacked A or B antigens present on the recipients' red cells. In case 6, the patient was A Rho(D) positive and the donor A Rho(D) negative; the donor's serum contained weak anti-D at the time of the transplant. All patients received cyclosporine after BMT to prevent graft-*v*-host disease (GVHD).

Group 2. To assess the frequency of immune hemolysis, a retrospective analysis of 97 consecutive BMT patients transplanted at one center (Hammersmith) was carried out. All patients received cyclosporine in the postgraft period for the prevention of GVHD. Twenty-one patients received minor ABO or D mismatched marrow (ie, donor lacking ABO or Rh antigens present in the recipient) and were designated "group 2" patients. Within group 2, 14 patients received marrow from donors lacking recipient A or B antigens; this included cases 1 and 2 from group 1. Seven D-positive recipients received marrow from D-negative donors and included case 6 from group 1. Two patients were ABO and D mismatched. Group 2 patient charts were reviewed for evidence of immune hemolysis. Weekly blood bank records from the post-BMT period were searched for the occurrence of any positive direct antiglobulin tests (DAT). Sera from group 2 patients receiving minor ABO mismatched marrow, stored at −20 °C for up to 36 months, were tested for donor type IgG and IgM anti-A and anti-B at one and three months after BMT.

Serology

Standard serological methods were used. The DAT was performed using polyspecific antihuman globulin (AHG) reagents. In DAT-positive cases, testing with anti-C3 and anti-IgG reagents was carried out. Eluates were made by the chloroform[1] or ether[2] technique. Anti-A and anti-B were estimated by titrating the stored

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Table 1. Clinical and Laboratory Data—Patients Presenting With Immune Hemolysis—Group 1

Case No.	Diagnosis	Age/Sex		ABO Group and Rh Type		Immunosuppressive Therapy		ABO Group and Rh Type of Transfusions		Details of Hemolysis and RBC Transfusion Requirement
		D	R	D	R	Pre-BMT	Post-BMT	Packed RBCs	Platelets	
1	CGL	17/M	15/F	O+	A−	Cy 60 mg/kg × 2 DN 60 mg/m² × 1 TBI 200 × 5 cGy	CyA HDMP	A− O−	A−	Severe IVH. Hemoglobinuria. Maximum hemolysis day +15. Treated with plasma exchange. Nine units RBCs required day +10 to +15.
2	CGL	13/M	11/F	O+	A+	Cy 60 mg/kg × 2 DN 60 mg/m² × 1 TBI 200 × 5 cGy	CyA HDMP	A+	A+	Moderate hemolysis, maximum day +16. Six units RBCs required day +16 to +22.
3	SAA	24/M	16/F	O+	B+	Cy 50 mg/kg × 4 TNI 750 cGy	CyA MP	O+	B+ O+*	Severe hemolysis. Hemoglobinuria. Maximum day +10. Fourteen units RBCs required day +10 to +19 (see Fig 1).
4	AML	11/F	14/M	O+	A+	TBI 120 × 11 cGy VP-16 40 mg/kg × 1	CyA MP	A+ O+	A+	Moderate hemolysis, maximum day +10. Seven units RBCs required day +9 to +18.
5	ALL	26/M	22/M	B+	AB+	Cy 120 mg/kg × 1 TBI 120 × 11 cGy	CyA MP	O+ B+	B+	Moderate hemolysis, maximum day +12. Six units RBCs required day +11 to +14.
6	SAA	32/F	17/F	A−	A+	Cy 50 mg/kg × 4 ALG 12.5 mg/kg × 4	CyA HDMP Azathiaprine	A−	A+ A−	Moderate hemolysis, maximum day +13. Five units RBCs required day +9 to +16.

*Platelets were washed and resuspended in AB plasma. D, donor; R, recipient; Cy, cyclophosphamide; DN, daunorubicin; TBI, total body irradiation; TNI, total nodal irradiation; CyA, cyclosporine; HDMP, high-dose methylprednisone; MP, methylprednisone; ALG, anti-lymphocyte globulin.

sera against suitable test red cells. The agglutination titer at 20 °C was measured as well as the titer at 37 °C using anti-IgG AHG in the indirect antiglobulin test. Appropriate saline controls were performed. The IgG and IgM components of the antibodies were estimated by testing the sera before and after treatment with 2-mercaptoethanol[3] or dithiothreitol.[4] The IgM titers were measured by agglutination at 20 °C, and the IgG titers were estimated by the indirect antiglobulin test at 37 °C.

RESULTS

Group 1. Group 1 patients presented clinically with hemolysis between days 9 and 16 post-BMT (median, day 10). The results of serological investigations are shown in Table 2. The DAT first became positive at four to 22 days (median, 12) after BMT. Antibodies against the recipient's red cell antigens were first detectable nine to 15 days (median, 13) after BMT. In cases 3 through 6 inclusive eluates prepared from recipient RBCs contained antibody to antigens present on recipient red cells of the same specificity found in the serum. Table 2 also shows that the red cell antibodies were only transiently present in the sera of the ABO-incompatible cases. However, in the D-incompatible case (case 6), the anti-D titer continued to rise, reaching a level of 256 on day 70 after BMT.

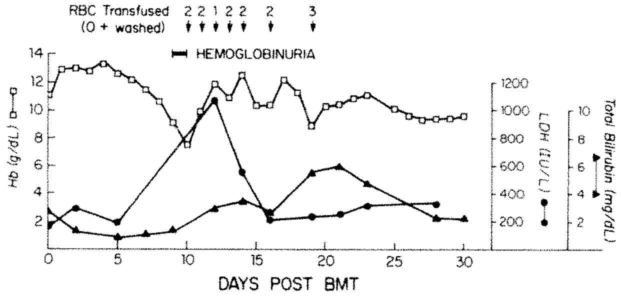

Fig 1. Case 3 from group 1. Minor ABO mismatched BMT (donor O+, recipient B+). Flow chart shows hematologic and biochemical evidence for hemolysis. Fourteen units of packed O+ cells were required to maintain the hemoglobin between day 10 and day 19 post-BMT. Note that the hemoglobin, the lactic dehydrogenase, and the total bilirubin were normal for the first five days post-BMT. During the hemolytic episode, no incompatible isohemagglutinins were infused; RBCs transfused were washed group O, platelets were group B or O washed and resuspended in fresh AB plasma.

Group 2. Relevant serological data is shown in Table 3. Six out of 16 patients receiving ABO mismatched marrow (including cases 1 and 2 from group 1) developed a positive DAT after BMT. However, only 2/6 (cases 1 and 2 in group 1) showed clinical evidence for immune hemolysis. Donor anti-A and anti-B titers pre-BMT and recipient anti-A and anti-B titers one and three months after BMT are shown. DAT-positive cases and clinical hemolysis could not be predicted by either donor or recipient antibody titers or the proportion of IgG and IgM antibody present. The antibodies were not tested with antisera specific for IgG subclasses.

Three out of the seven patients in group 2 who received D-incompatible marrow (including patient 6 from group 1) developed a positive DAT after BMT (Table 3). In all three DAT-positive cases, anti-D was detected in the serum after BMT. In one case (AG), anti-C and anti-E were also detected. Only one patient (AR) with a positive DAT due to rhesus antibodies (AR) showed clinical evidence of hemolysis (case 6 in group 1; see Tables 1 and 2).

DISCUSSION

Anti-A and anti-B production against donor red cells by residual *recipient* plasma cells after *major* ABO mismatched

Table 2. Serological Investigation of Patients Presenting With Immune Hemolysis—Group 1

Case No.	Maximum Hemolysis Day Post-BMT	ABO Group Rh Type D	ABO Group Rh Type R	DAT Positive for	Day Post-BMT DAT First Positive	Antibody in Eluate	Antibody in Serum	Day Post-BMT Serum Antibody Last Detected
1	+15	O+	A−	C3	+18	ND	Anti-A	+38*
2	+16	O+	A+	C3	+15	ND	Anti-A	+24*
3	+10	O+	B+	IgG + C3	+10	Anti-B	Anti-B	+42*
4	+10	O+	A+	C3	+4	Anti-A	Anti-A	+30*
5	+12	B+	AB+	C3	+9	Anti-A	Anti-A	+42*
6	+13	A−	A+	IgG	+13	Anti-D	Anti-D	+70†

DAT, direct antiglobulin test; D, donor; R, recipient; ND, not done.

*Serum antibody not detectable in subsequent follow-up.

†Anti-D titer 256; later samples not tested.

BMT is well documented.[5] Hemolysis of donor red blood cells at the time of the transplant can be prevented by removal of recipient antibodies by plasma exchange[6] or by removal of donor red cells from the marrow before infusion.[7,8]

More recently, cases of immune hemolysis occurring in recipients of *minor* ABO mismatched marrow have been reported.[9,10] Some reported episodes of hemolysis were clinically severe, and two cases of acute renal failure associated with hemoglobinuria were documented.[9] Cases of immune hemolysis occurring in patients receiving minor ABO mismatched liver[11] and kidney[12] transplants have also appeared in the literature.

We have investigated six patients from three transplant centers who presented with immune hemolysis after minor ABO or D mismatch BMT. From our data, active production of donor-derived red cell antibody is the most likely mechanism. Maximum hemolysis occurred one to three weeks after BMT, suggesting that passive transfer of antibody at the time of the transplant is an unlikely cause. None of the patients in group 1 received significant ABO-incompatible plasma in blood products (Table 1); therefore, transfusion of incompatible plasma does not account for the phenomenon.

Retrospective analysis of minor ABO mismatched BMT patients showed that only a minority (2/16) developed clinically significant hemolysis, despite the production of antibody against recipient red cell antigens in the majority of cases (Table 3). Six out of 16 patients developed a positive DAT, suggesting that in vivo red cell sensitization often occurs without signs of overt hemolysis. From data shown in Table 3, it can be seen that the onset of immune hemolysis cannot be predicted from either donor or recipient anti-A or anti-B titers. Most minor ABO mismatched patients were transfused with packed group O cells. However, the transfusion of group A or B red cells may have contributed to the severity of hemolytic episodes seen in patients 1, 2, 4, and 5 in group 1 (Table 1).

We have shown that production of donor-derived antibody specific for recipient ABO antigens is transient, with the relevant antibody being virtually undetectable by three months post-BMT (Table 3). These data are consistent with a report by Buckner et al[6] in which donor-derived antibody against recipient ABO antigens was absent or weak one year after BMT. The transient nature of donor-derived antibody against recipient ABO antigens is not understood and contrasts with the more persistent donor-derived rhesus antibodies detected. Possible mechanisms are discussed below.

The probable cause of immune hemolysis in case 6 group 1 was donor-derived anti-D. Anti-D was not detectable in the recipient's serum until 13 days after BMT and rose to a titer of 256 on day 70 after BMT. On retrospective analysis of 97 cases, seven D-positive patients received marrow from D-negative donors. Three out of seven patients (including patient 6 in group 1) produced donor-derived anti-D (Table

Table 3. ABO Minor and Rhesus (D) Mismatch BMT (Group 2)

Patient	Relevant Antibody	Donor Antibody Titer IgG/IgM	Recipient Antibody Titer 1 mo Post-BMT IgG/IgM	Recipient Antibody Titer 3 mo Post-BMT IgG/IgM	DAT Day Positive Post-BMT
AG	Anti-B	64/2	4/2	<2/<2	+28
JC	Anti-B	32/16	4/2	<2/<2	0
CW	Anti-B	32/8	4/2	<2/<2	+7
CR	Anti-A	32/32	2/2	<2/<2	+4
AK	Anti-A	32/64	8/2	<2/<2	+22
DD	Anti-B	64/128	<2/<2	<2/<2	+8
PG*	Anti-A	128/32	8/4	ND	+22†
KS	Anti-A	ND	ND	ND	0
SS	Anti-B	ND	ND	ND	0
GB	Anti-A	64/16	8/2	<2/<2	0
JW‡	Anti-A	16/4	8/2	2/<2	+19†
NW	Anti-A	16/32	4/4	<2/<2	0
KR	Anti-B	16/2	8/2	<2/<2	0
SR	Anti-A	16/8	2/<2	<2/<2	0
MT§	Anti-A Anti-D	64/64	4/4	4/<2	0
JR§	Anti-B Anti-D	ND	ND	ND	0
AR‖	Anti-D	Anti-D¶	4	256	+13†
CJ	Anti-D	0	Anti-D¶	Anti-D¶**	+22
AG	Anti-D	0	Anti-D¶ Anti-C¶ Anti-E¶	Anti-D¶** Anti-C¶ Anti-E¶	+21
CH	Anti-D	0	0	0	0
TP	Anti-D	0	0	0	0

ND, not done.

*Case 2 from group 1.

†Clinically significant hemolysis.

‡Case 1 from group 1.

§Minor ABO *and* rhesus (D) mismatch. No anti-D detected in recipients.

‖Case 6 from group 1.

¶Titer not estimated.

**Anti-D last detected on day +98 (CJ) and +365 (AG).

3). In contrast to the transient antibody production in the ABO cases, the recipients of D-incompatible marrow continued to produce anti-D for prolonged periods of up to one year after BMT. The early disappearance of donor-derived antibodies in the ABO cases may be due to high zone tolerance brought about by continued exposure of donor lymphocytes to A or B antigens in the recipients' plasma and tissues. In contrast, the rhesus mismatched BMT donor lymphocytes are only exposed to recipient rhesus antigens transiently until residual recipient red cells are eliminated from the circulation. The antigenic stimulus for donor-derived rhesus antibody production may not be strong enough to induce tolerance, but instead leads to prolonged antibody production.

The role of cyclosporine in facilitating donor-derived antibody production is uncertain. It is of interest that all cases of immune hemolysis due to donor-derived antibody so far reported have been in transplant recipients immunosuppressed with cyclosporine. Our own experience confirms this observation. We have retrospectively reviewed 13 patients transplanted in one center (Duarte) in whom methotrexate was used in the postgraft period to prevent GVHD (data not shown). We have been unable to identify a single case of immune hemolysis due to donor-derived antibody. This group of patients is now being studied prospectively to confirm our retrospective observations.

Cyclosporine is a powerful immunosuppressive agent that acts primarily by blocking proliferation of T lymphocytes in response to antigen or mitogens.[14,15] However, T lymphocyte proliferation is not impaired when presensitized lymphocytes are reexposed to antigen in the presence of cyclosporine.[16] In minor ABO mismatched BMT, the donor lymphocytes are sensitized in vivo before transfer to the recipient, so donor-derived red cell antibody production is a secondary immune response. In one D minor mismatched case (AR), we have shown that the donor was presensitized to the D antigen, with anti-D present in the donor's serum. The donor-derived anti-D produced in this recipient was, therefore, the result of a secondary immune response to the D antigen by the donor lymphocytes, analogous to the production of anti-A and anti-B in the ABO-incompatible cases described. We have no definite evidence for donor presensitization to rhesus antigens in the other two cases in which donor-derived rhesus antibodies were produced. Indeed, the donor for patient AG was a 15-year-old boy who had not been transfused, suggesting that in this case, the production of donor-derived anti-D, -C, and -E in the recipient was due to a primary immune response by the donor lymphocytes. In the third case in which donor-derived rhesus antibodies were detected (CJ), the marrow was donated by the patient's sister. Her serum did not contain detectable rhesus antibodies at the time of the transplant; however, she has two rhesus D-positive children aged 15 and 17 years. It is therefore possible that the donor became sensitized to the D antigen during pregnancy and that the production of donor-derived anti-D in the recipient was a secondary response.

Although the frequency of clinical hemolysis is low in minor ABO and rhesus mismatched BMT, it can be severe. All six group 1 patients required increased red cell support, two patients had evidence for intravascular hemolysis, and one patient required plasma exchange. Renal failure was not apparent in our patients; however, this remains a potential complication. It is not possible to predict which patients will hemolyze from monitoring donor and recipient anti-A and anti-B titers (Table 3). Our data suggest that significant hemolysis occurs in approximately 10% to 15% of cases receiving minor ABO or D mismatched transplants. To prevent exacerbation of hemolysis, recipients of minor ABO mismatched transplants should be transfused with packed or washed group O red cells. In D mismatched cases, D-negative red cells of the appropriate ABO group should be given. Platelet concentrates given to minor ABO mismatched BMT patients should be *recipient* group or group O. If group O platelets are used, transfusion of significant ABO-incompatible plasma can be avoided by using platelet preparations concentrated by centrifugation.[17] These measures will, however, not completely prevent hemolytic episodes.

In summary, we have documented the occurrence of immune hemolysis in approximately 10% to 15% of marrow transplant recipients who received minor ABO or D mismatched marrow and were treated with cyclosporine. We believe that hemolysis is due to active production of red cell antibody by donor lymphoid cells in the early posttransplant period. Antibody production may be associated with cyclosporine immunosuppression. Further work is in progress to evaluate the role of cyclosporine in the pathogenesis of this syndrome. When hemolysis occurs, it may be severe, and we have suggested a transfusion policy for patients receiving minor mismatched transplants that we hope will prevent exacerbation of the hemolysis.

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Diaziquone Given as a Continuous Infusion Is an Active Agent for Relapsed Adult Acute Nonlymphocytic Leukemia

By Edward J. Lee, David A. Van Echo, Merrill J. Egorin, M.S. Balchandran Nayar, Philip Shulman, and Charles A. Schiffer

Diaziquone given as a bolus has not been effective in patients with relapsed or refractory leukemia. Because of in vitro data suggesting enhancement of diaziquone-induced cytotoxicity for human and murine leukemia cells with increased duration of drug exposure and the relatively short terminal plasma half-life of diaziquone, 49 patients (34 acute nonlymphocytic leukemia [ANLL], six chronic myelogenous leukemia in blast crisis [CML-B], five acute lymphocytic leukemia [ALL], four 2° ANLL) with leukemia were given diaziquone as a continuous infusion for seven days. The maximum tolerated dose was 28 mg/m^2/d for seven days. The dose-limiting toxicity was the duration of bone marrow aplasia (median, 49 days to >500 PMNs in responders; range, 28 to 101 days). Nonhematologic toxicity was minimal. Responses occurred only in patients with relapsed ANLL, of whom 26 were treated at effective doses. There were six complete responses (CR) (23%) and two partial responses (PR) (8%), although five of eight responders never achieved platelet counts >100,000/μL. Thrombocytopenia in these patients was felt to be a manifestation of diaziquone effect, not persistence of leukemia. The median duration of CR was 195 days (range, 88 to 860+). One patient had active CNS leukemia at the start of treatment and has had a durable (28+ month) CR in both sites of disease. Diaziquone produced prolonged aplasia in patients with secondary ANLL and CML-B (five of ten patients died aplastic), whereas patients with ALL all had regrowth of leukemia and two failed to become aplastic. The lack of significant nonhematologic toxicity and the activity in patients with relapsed ANLL render diaziquone of interest as second-line therapy or consolidation therapy in first remission for patients with ANLL.

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COMBINATION CHEMOTHERAPY with an anthracycline and cytosine arabinoside has resulted in a substantial improvement in the treatment of adult acute nonlymphocytic leukemia (ANLL). Complete response (CR) rates of 60% to 80% and median survival of 18 to 30 months have been reported.[1-6] Nonetheless, the majority of patients who achieve remission, relapse, and as many as 20% fail to achieve an initial remission. Thus, a need exists for other agents that may be non-cross-resistant with the standard combinations for use either in induction or consolidation regimens.

Diaziquone (AZQ, NSC182986) is a rationally synthesized aziridinylbenzoquinone compound with good lipid solubility and low ionization allowing access to the central nervous system.[7] Diaziquone has activity in vitro against the murine leukemia cell lines L1210 and P388 and against the human leukemia cell line HL-60. In murine systems, in vivo activity against intracranially and intraperitoneally implanted tumors is seen when diaziquone is given by way of an intraperitoneal route.[8] Phase I studies using a bolus schedule in solid tumor and leukemia patients showed that diaziquone produced primarily hematologic toxicity with minimal nonhematologic side effects.[9] No responses were observed in patients with leukemia.[10-12] Pharmacokinetic studies showed that diaziquone was cleared rapidly from the plasma with a terminal half-life of approximately 30 minutes.[9,13] In vitro studies from our center demonstrated that cytotoxicity against L1210 and HL60 leukemia cell lines could be enhanced by increasing the duration of exposure of the cells to diaziquone.[14] On the basis of this demonstration of schedule-dependent enhancement of cytotoxicity, a continuous infusion trial in patients with solid tumors was carried out at the University of Maryland Cancer Center and showed no change in the known toxicities.[15] As the only toxicity of diaziquone using both bolus and continuous infusion schedules was myelosuppression, a continuous infusion phase I/II study of diaziquone was designed for patients with relapsed or refractory adult leukemia.

MATERIALS AND METHODS

Patients. Eligible patients included the following adults (age >15 years): (a) patients with de novo ANLL, either relapsed (those who had had a CR to other therapy) or refractory (those who had never achieved a CR); (b) patients with ANLL developing after a previous hematologic disorder or treatment of another malignancy who had already received combination chemotherapy with anthracycline/cytosine arabinoside (2° ANLL); (c) patients with relapsed acute lymphocytic leukemia (ALL); (d) patients with chronic myelogenous leukemia in blast crisis (CML-B).

All patients had their diagnoses confirmed by bone marrow aspirate or biopsy, or both when possible. Other eligibility criteria included recovery from previous chemotherapy or radiation therapy, a minimum life expectancy of six weeks, and adequate hepatic (bilirubin <1.5 mg/dL) and renal (creatinine <1.5 mg/dL) function. All patients had either a normocellular bone marrow with at least 30% leukemic cells or evidence of progressive leukemia. This was defined as increasing numbers of leukemic cells in the peripheral blood accompanied by signs of bone marrow failure, such as anemia, granulocytopenia, or thrombocytopenia. Written informed consent was obtained from each patient before entry onto study.

During the seven-day treatment period, all patients were examined at least daily. Complete blood counts and platelet counts were obtained daily; laboratory profiles, including hepatic and renal function, were obtained twice. After the chemotherapy period, patients had bone marrow aspirates and biopsies at weekly intervals. Hematologic, hepatic, and renal parameters were monitored on at least a weekly basis and more often as clinically indicated.

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The bone marrow response determined whether a second course of diaziquone was given. If the marrow revealed increasing cellularity with persistence of leukemia after day 15, then a second course of diaziquone was given. No further chemotherapy was given if remission was achieved. Patients who failed to achieve a response to a second course were taken off study.

Responses were judged according to the following criteria: a CR was defined as an M-1 bone marrow (a normocellular marrow with <5% leukemic cells, normal maturation of the granulocyte and erythroid cell lines, and the presence of megakaryocytes) and no evidence of extramedullary disease. All patients considered to be in CR were required to have normal peripheral granulocyte counts (>1,500/μL) and to have no need for red cell or platelet transfusions. A "normal" platelet count was not required because diaziquone has been documented to cause prolonged thrombocytopenia.[16] Thus patients who were felt to have thrombocytopenia due to the drug with marrows that were otherwise M-1 were designated to be in CR. A partial response (PR) was defined as a normocellular bone marrow with fewer than 5% blasts and fewer than 30% blasts plus progranulocytes according to the criteria of the Cancer and Acute Leukemia Group B (CALGB). Nonhematologic toxicity was assessed according to the criteria of CALGB on a scale from 0 to 4.

Supportive care for all patients consists of oral prophylactic antibiotics[17]; empiric or specific parenteral antibiotics for documented or suspected infections; prophylactic platelet transfusions[18]; granulocyte transfusions for documented infections not controlled by appropriate antibiotics.[19]

Drug schedule and drug administration. Diaziquone was supplied in 10-mg vials by the Investigational Drug Branch of the National Cancer Institute (NCI) and was reconstituted with sterile *N,N*-dimethyl-acelanide and phosphate buffer for injection according to NCI recommendations. The total daily dose of diaziquone was diluted in 1,000 mL of normal saline and was prepared within six hours of use. Bottles were changed every 24 hours, since diaziquone in normal saline at the concentrations used undergoes 10% decomposition at 44 hours. The drug was administered as a continuous infusion for seven days (with two exceptions noted later) by infusion pump.

Cohorts of two to three patients were treated at doses ranging from 16 mg/m^2/d to 32 mg/m^2/d. The initial dosage was twice the maximum tolerated dose (MTD) defined in our phase I continuous infusion trial of diaziquone in solid tumor patients,[15] but 2/3 of the MTD in the phase I bolus leukemia study performed in our center.[10] Dose escalations were 4 mg/m^2/d and patients who failed to respond to their initial therapy were eligible to receive a second course at the next higher dose.

Pharmacokinetic studies. Blood samples were collected in heparinized tubes, before institution of diaziquone infusion, at 5, 15, 30, 45, 60, and 120 minutes and 24, 48, 72, 96, 120, 144, and 168 hours into the infusion and at 2, 5, 10, 15, 30, 45, and 60 minutes after termination of the infusion. Samples were centrifuged at 1,000 g for ten minutes, and the resulting plasma was removed and frozen at −20 °C until assay. Diaziquone concentrations in plasma were determined with the high-performance liquid chromatography analysis of Kelley and Siu Chong.[20] Pharmacokinetic modeling was accomplished with the MLAB Program.[21]

RESULTS

Forty-nine patients with relapsed or refractory adult leukemia were entered onto this study (Table 1). Patients with ALL had all received standard induction regimens initially and received diaziquone after other second-line treatments were unsuccessful. Diaziquone was the initial treatment in five of six individuals with CML-B; the other is described below. All patients with 2 ° ANLL were treated initially with daunorubicin and cytosine arabinoside in conventional doses. Diaziquone was the second induction regimen used in each of these patients. In patients with de novo ANLL, 11 patients had had two or more prior CRs. Diaziquone represented from the third to the tenth induction regimen given to these patients (median, three prior regimens). There were 18 patients with ANLL who had had a single prior CR. Of these, 11 received diaziquone as their initial treatment for relapse. Seven patients had undergone reinduction with either conventional-dose anthracycline/cytosine arabinoside (three patients) or high-dose cytosine arabinoside (four patients) and received diaziquone after these efforts were unsuccessful.

The number of patients treated at each dosage and the treatment outcome is presented in Table 2. No patients with ALL responded. Although all patients became hypocellular, two were never aplastic and all had regrowth of leukemia. Six patients with CML-B were treated. One was treated at 24 mg/m^2/d for seven days. This individual had a lymphoid blast crisis treated with vincristine and prednisone with an excellent response, followed by allogeneic bone marrow transplantation. Bone marrow relapse with associated hypercalcemia, lytic bone lesions and bone pain developed a few

Table 1. Patient Characteristics

	Diagnosis (n = 49)			
	ANLL (de novo) (n = 34)	ANLL (2°) (n = 4)	CML-B (n = 6)	ALL (n = 5)
Age–median	44	47	39	30
(range)	(16–72)	(40–69)	(23–48)	(19–60)
Sex				
M	21	1	5	4
F	13	3	1	1
Performance status (CALGB)				
0	19	1	3	2
1–2	10	1	2	2
3–4	5	2	1	1
Prior CR ≥2	11	0	1	2
1	18	2	0	3
0	5	2	0	0
No prior treatment	0	0	5	0

Table 2. Response to Diaziquone by Disease and Dosage

Disease	Dosage	Days	No. of Patients	CR	PR	Died Aplastic	Never Aplastic
ANLL (de novo)	16 mg/m^2	7	3	0	0	1	1
	20	7	2	1	0	0	0
	24	7	1	0	0	0	0
	28	5	1	0	1	0	0
	28	7	26	5	1	3	2
	32*	7	1	0	0	1	0
TOTAL			34	6	2	5	3
ANLL (2°)	28	7	4†	0	0	2	0
CML-B	24	7	1	0	0	0	0
	28	5	1	0	0	0	0
	28	7	4	0	0	3	0
ALL	28	7	4	0	0	0	2
	32*	7	1	0	0	1	0

*Both trials complicated by bone marrow necrosis.

†One patient inevaluable for response because of anaphylaxis.

months later. Diaziquone was administered and the symptoms abated only transiently. Four patients with CML-B received 28 mg/m^2/d for seven days. Three died aplastic (days 34, 43, and 65) and one had disease progression after prolonged aplasia (day 51). Because of the prolonged aplasia, the sixth patient was treated for five days at 28 mg/m^2/d and failed to achieve aplasia. Three patients with secondary ANLL were treated at a dosage of 28 mg/m^2/d for seven days. No responses were seen. All three patients became aplastic. Of these, two died aplastic (days 29 and 40) and one developed progressive leukemia. A fourth such patient was to have received 28 mg/m^2/d for seven days but is inevaluable for response due to an anaphylactic reaction within minutes of starting diaziquone. The infusion was stopped and not restarted.

Thirty-four patients with relapsed (29) or refractory (five) de novo ANLL were treated at varying doses of diaziquone. Six CRs and two PRs were seen in the relapsed ANLL group. Five patients in this group died aplastic without evidence of leukemia in the marrow at the time of death (days 15, 28, 38, 38, 44). Two patients failed to achieve aplasia (one at 16 mg/m^2/d and the other at 28 mg/m^2/d), and 14 became aplastic but had regrowth of a leukemic bone marrow. Responses occurred at dosages between 20 and 28 mg/m^2/d for seven days except for one patient who received only a five-day infusion (due to patient refusal to continue treatment although no toxicity was apparent). This individual had rapid regeneration of normal blood counts by day 28 but did not return to the hospital until two months later, when relapse was apparent. A bone marrow aspirate on day 28 showed an M-2 bone marrow with maturation in all three cell lines. No further specimens could be obtained until the patient returned with obvious progressive leukemia. The other PR occurred in an elderly patient with a regenerating bone marrow with <5% blasts and normal granulocyte counts, who died of infectious complications on day 48. All responders had slow bone marrow recovery (Table 3). Of note in these responders is that five of the eight never had platelet counts over 100,000/μL, although all but one were greater than 75,000/μL and none were transfusion dependent. This will be discussed in detail later. Thus, the overall CR rates in patients with de novo ANLL treated at what may represent effective doses ($\geq$20 mg/m^2) is 6/31, or 19% (95% confidence limits 5% to 32%). The CR rates were 6/26, or 23% (95% confidence limits 7% to 39%), if patients with primary refractory ANLL are excluded (Table 4).

Three patients had remission durations from diaziquone that exceeded the length of their prior remission durations (eight months compared with CR of six months to each of two prior regimens; 28+ months compared with 17 months for a single prior CR; 16+ months compared with eight months for a single prior CR). The second patient merits a more detailed description. She developed ANLL (FAB M-4) at age 53 and was treated with conventional-dose daunorubi-

Table 3. Responders

Patient/Response	Dosage (mg/m^2/d × No. of Days)	Duration of Response (d)	Days* To	
			>500 PMN	Last Platelet Transfusion
JS/CR	20 × 7	88	48	52
AW/CR	28 × 7	108	49	47
TP/CR	28 × 7	860+	101	91
EA/CR	28 × 7	250	46	38
MP/CR	28 × 7	140	54	28
RK/CR	28 × 7	485+	49	40
BB/PR	28 × 7	8	40	47
KM/PR	28 × 5	71	27	27

*Measured from first day of treatment with diaziquone.

Table 4. De Novo ANLL at Effective Doses (20–32 mg/m^2/d)

	No. of Patients	CR	PR	Resistant Disease	Died Aplastic
Relapsed	26	6	2	14	4
Refractory	5	0	0	5	0

cin and cytosine arabinoside with a CR that, with cytosine arabinoside/6-thioguanine intensification every 10 weeks, lasted for 17 months. On relapse, she was unsuccessfully retreated with daunorubicin/cytosine arabinoside in the same doses amd developed CNS leukemia during bone marrow recovery. She received cranial irradiation and intrathecal methotrexate but still had leukemic cells in her cerebrospinal fluid and focal symptoms when she was treated with diaziquone. Without further specific CNS therapy, the symptoms and findings of CNS leukemia cleared, and despite prolonged aplasia (101 days to >500 PMN, 91 days to last platelet transfusion), she achieved a complete remission and continues in unmaintained CR 32 months after initiation of diaziquone.

No responses were seen in the group of five patients who had no prior CR. Four became aplastic before developing progressive leukemia; one did not become aplastic.

Five patients received a second course of diaziquone. Two were escalated from the first two dose levels (16 to 20 mg/m^2/d, 20 to 24 mg/m^2/d); the third had a CR at 20 mg/m^2/d and was retreated at 28 mg/m^2/d after relapse. The fourth was treated initially at 28 mg/m^2/d. After an uneventful but unsuccessful course, he received a second course at 32 mg/m^2/d. The fifth patient had a transient improvement in his bone marrow after an initial course and received a second course at the same dose (28 mg/m^2/d). None of these patients responded to the second course.

Toxicity. Nonhematologic toxicity was mild (Table 5). Grade 3 gastrointestinal toxicity occurred in three patients (vomiting, two patients; mucositis, one patient), and no grade 4 toxicity was noted. Hepatic toxicity occurred in 21% of all courses and was grade 1 (ten patients) or grade 2 (one patient). Several patients had marked abnormalities in liver function late in their courses associated with infection and multiorgan system failure, usually as a preterminal event.

Of the 11 patients who died aplastic, ten died of infectious consequences and one alloimmunized patient died of an intracranial hemorrhage on day 15. There were no drug-related toxicities involving the central nervous, renal, cardiac, or pulmonary systems. One patient had an anaphylactic reaction to diaziquone within minutes of starting the initial infusion. She had a history of multiple drug allergies and had had an anaphylactic reaction to vitamin K two weeks before institution of diaziquone therapy. Her reaction (bronchospasm, flushing, hypotension) was successfully treated without complications. She received no further diaziquone.

Table 5. Nonhematologic Toxicity

Dosage (mg/m^2/d)	No. of Patient Courses	Vomiting	Mucositis	Hepatic
16	3	2	0	0
20	3	1	0	0
24	3	1	0	0
28 × 5 d	2	0	0	1
28 × 7 d	39*	16 (2)	8 (1)	10
32	3	0	1	0
	53			

All grade 2 or less except those in parentheses (grade 3 or 4).

*One patient not included here developed anaphylaxis within minutes of initiation of diaziquone therapy.

Three courses of therapy were given at 32 mg/m^2/d for seven days. In one patient with ANLL, bone marrow necrosis occurred. This individual had, at the outset, a performance status of 3, significant bone pain with a markedly hypercellular bone marrow, extramedullary disease (large subcutaneous chloromas), and infection. After the initiation of treatment with diaziquone, his bone pain resolved within 24 hours and his chloromas resolved within a week. Bone marrow aspirates and biopsies from multiple sites showed necrotic material throughout his course. No regeneration of normal marrow elements or leukemic cells occurred and death occurred after 38 days. A second individual with ALL also developed bone marrow necrosis but thereafter developed progressive leukemia after 30 days. A third patient had an uncomplicated but unsuccessful course at 32 mg/m^2/d after an initial course of diaziquone at 28 mg/m^2/d.

Hematologic toxicity was profound. All patients required red blood cell and platelet transfusions. The dose-limiting toxicity of diaziquone was the duration of aplasia (median, 49 days to >500 PMN for responders; range, 27 to 101). In view of this and the occurrence of bone marrow necrosis in two of three patients at 32 mg/m^2 for seven days, no further courses at dosages above 28 mg/m^2/d for seven days were given.

Pharmacokinetic studies. Steady-state plasma concentrations of diaziquone in 11 patients treated with 28 mg/m^2/d for seven days were 101 ± 10 ng/mL (mean ± SEM; range, 54 to 150 ng/mL) and were achieved 45 minutes after initiating treatment. At the cessation of the infusion, plasma concentrations of diaziquone declined by monoexponential decay with a mean half-life of 47.7 ± 11.4 minutes. Little variability was seen in steady-state concentrations in individual patients.

DISCUSSION

Previous studies of diaziquone given as a bolus have not demonstrated activity either in ANLL or in ALL.[10–12] Fifty-seven patients (36 with ANLL) have been treated in these studies with no CR or PR reported. On the basis of observations of enhanced cytotoxicity against leukemia cell lines with increased duration of exposure to diaziquone at our center, a continuous infusion trial has been conducted. When given by this method, a significant level of activity was seen in patients with de novo ANLL who had achieved a prior remission. In this group, 26 patients were treated at doses that produced responses, and six achieved CR (23%) and two achieved PR (8%) for an overall response rate of 31% (8/26) (Table 5). This strongly suggests that the change in drug scheduling was responsible for these improved results. These response rates compare favorably with response rates for other new agents, such as m-AMSA,[22] homoharringtonine,[23] and mitoxantrone.[24]

Five of the responders failed to regenerate platelet counts in excess of 100,000/μL. Examination of several bone marrows in each of these patients revealed fewer than 5% blasts, normal myeloid and erythroid maturation, and diminished numbers of megakaryocytes. One responder had biopsy-proven chronic active hepatitis that antedated diaziquone, splenomegaly, and a platelet count of 88,000/μL 16 months after treatment. Because diaziquone is associated with prolonged thrombocytopenia in patients with solid tumors, it is our feeling that failure to regain a normal platelet count is drug-related and not evidence of persisting leukemia. In the patient who had the best response (28+ months), a year passed before the platelet count became normal (>150,000); currently, it is between 250,000 and 300,000/μL. Thus, despite the lack of a normal platelet count, we feel that these individuals with otherwise normal-appearing bone marrows, diminished megakaryocytes, and no transfusion requirement should be considered CRs.

Prolonged bone marrow aplasia was seen in all patients with CML-B and 2 ° ANLL treated for seven days, but no responses occurred. Of the eight patients treated for seven days, five died aplastic. In these two groups with possibly depleted stem cell pools, it may be necessary to reduce the dosage of diaziquone. In patients with ALL, the failure to achieve aplasia in conjunction with the toxicity at 32 mg/m^2/d make it likely that diaziquone will not have much impact on the treatment of ALL. Although an insufficient number of patients are reported to exclude activity, no responses were seen.

Nonhematolgic toxicity was minimal. Anaphylaxis was seen in one patient. Although this complication has been reported,[25] it has, in the past, only been associated with individuals who had prior exposure to the drug. This patient had no such prior exposure, although a recent reaction to a drug with similar structure and a history of multiple drug allergies was present. Diaziquone should be administered with caution to patients with similar histories.

Bone marrow necrosis occurred in two of three patients given 32 mg/m^2/d. Although other causes exist that may cause this complication, such as leukemia and infection,[26] the prolonged duration of aplasia at 28 mg/m^2/d suggests that further dose escalations will not be possible in patients with ANLL. Approximately ¼ of the patients given full-dose diaziquone developed mild mucositis. Symptoms were limited to the mouth. Of note is the lack of significant hepatic toxicity. In previous reports involving diaziquone, two patterns of hepatic abnormalities were described. One consisted of a transient minimal elevation of transaminases or bilirubin. The other consisted of marked abnormalities occurring late in the course associated with systemic infection. The first pattern is likely to be drug-related but of little clinical consequence, whereas the second pattern is not clearly related to diaziquone and is generally a preterminal event. Both patterns were observed in the patients in this study, yet two patients with biopsy-proven chronic active hepatitis received full-dose diaziquone (28 mg/m^2/d for seven days) without worsening of hepatic function. One of those individuals died aplastic (day 42). The other achieved a CR with bone marrow recovery after six weeks similar to other responders.

Several patients, responders and nonresponders alike, had benign courses with minimal or no infectious complications and were managed as outpatients for much of their course. This lack of early complications is unique to diaziquone and unlike any other phase II agent studied in relapsed leukemia, such as anthracycline analogues,[24] high-dose cytosine arabinoside,[27] homoharringtonine,[23] or etoposide.[28] The prolonged period of granulocytopenia and infectious consequences represents the dose-limiting toxicity.

The steady-state plasma concentration of diaziquone achieved in this study agreed with those predicted from the relationship:

$$\text{Steady-state concentration} = \frac{\text{Rate of infusion}}{\text{Total body clearance}}.$$

From the rate of infusion of 28 mg/m^2/d and the total body clearance values of 517 ± 159 mL/min defined in the studies of intravenous bolus diaziquone,[9,13] plasma steady-state diaziquone concentrations of 49 to 92 ng/mL would be expected. Not only are the calculated values similar to those actually measured in our patients (range 54 to 150 ng/mL), but they are also in the range of diaziquone concentrations ($IC_{50} \approx 100$ ng/mL) shown by us to inhibit the in vitro growth of human and murine leukemia cells.[14] In addition, the favorable response seen in the only patient with leptomeningeal leukemia is compatible with the observation that administration of diaziquone by continuous infusion to dogs produces drug concentrations in the CNS equivalent to those in the plasma.[29]

The plasma terminal half-lives of diaziquone measured in patients treated in this study are somewhat longer than the 31- and 33-minute half-lives observed after intravenous bolus administration of diaziquone.[9,13] It is possible that the slightly longer half-lives and somewhat higher steady-state diaziquone concentrations observed in our patients reflect some subclinical hepatic effect, such as reduced metabolism or clearance associated with prolonged infusion of high concentrations of diaziquone. On the other hand, it may not be necessary to implicate any such alterations, since there is reasonable agreement between the pharmacokinetic values measured and defined in this study and those described previously in studies using strikingly different doses and schedules.

The lack of extramedullary or liver toxicity make diaziquone an interesting drug to combine with another agent of established activity in the treatment of ANLL. Indeed, both the duration of response and the occurrence of a durable CR in a patient who had failed to achieve remission after reinduction with daunorubicin and cytosine arabinoside render diaziquone of interest as either second-line reinduction therapy or as consolidation after initial remission because of the potential lack of cross-resistance. However, in view of the prolonged duration of bone marrow aplasia, it will be necessary to reduce either the dosage or the duration of infusion of diaziquone for use in combination. A trial of diaziquone and amsacrine in combination is in progress at our center.

The demonstrated activity and mild nonhematologic toxicity also raise the possibility of using diaziquone in preparative regimens for bone marrow transplantation for patients with ANLL. Resistant leukemia remains a major problem in patients transplanted in relapse or first or second remission who have at least a 15% chance of developing leukemia after transplantation.[6] As bone marrow necrosis occurred when the dose of diaziquone was increased, it may not be possible to treat patients at higher dosages than that defined in this study. Nonetheless, it should be noted that alternative explanations for bone marrow necrosis existed. Studies to define the role of diaziquone in this setting would be of considerable interest.

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Modulation of Human Neutrophil Effector Functions by Monoclonal Antibodies Against Surface Membrane Molecules of 94,000 and 180,000 Molecular Weight

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Function-related antigens on the neutrophil (PMN) surface were identified using two newly developed PMN-specific mouse monoclonal antibodies. These IgG antibodies, designated Ab 1-14 and Ab 1-15, were selected for detailed study after initial testing revealed their significant inhibition of PMN superoxide generation in response to *N*-formyl-Met-Leu-Phe (FMLP) (64% for 1-14 and 64% for 1-15; $P < .05$). In further experiments, Ab 1-14 augmented PMN adhesion (by 111%; $P < .01$) and degranulation (by 52%; $P < .05$) in response to FMLP, while Ab 1-15 inhibited these responses by 42% and 29%, respectively ($P < .05$). Ab 1-14 reduced PMN chemotaxis in response to FMLP by 37% ($P < .02$), and unlike Ab 1-15, Ab 1-14 significantly reduced unstimulated PMN binding of complement-coated sheep red blood cells. Ab 1-14 and Ab 1-15 significantly reduced PMN superoxide production in response to phorbol myristate acetate (PMA) (14% and 23%, respectively; $P < .05$). Whereas 1-14 was found to increase PMA-induced cell degranulation significantly (175%), Ab 1-15 did not alter degranulation response to PMA. Immunoprecipitation showed that Ab 1-14 and Ab 1-15 recognized respective surface antigens of 94,000 mol wt and 130,000 to 180,000 mol wt. Our findings suggest that the surface molecules identified by these two monoclonal antibodies play a significant role in neutrophil activation by both FMLP and PMA.

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CELL SURFACE INTERACTION with external stimuli is required for in vitro activation of neutrophil (PMN) functions, including chemotaxis, adhesion, and secretion.[1-8] Because chemical and biochemical techniques have been unable to isolate single, well-defined functional molecules from complex PMN surface structures, the roles played by various surface components in the PMN activation sequence remain poorly defined.

In the present study, monoclonal antibodies were used to identify individual PMN surface structures and to study the role of these molecules in PMN activation. We developed antineutrophil-specific murine monoclonal antibodies and screened these reagents for modulation of PMN functions in vitro. Two anti-PMN monoclonal antibodies were identified that significantly altered PMN function in studies of cell adherence, locomotion, superoxide generation, and degranulation. Binding of individual antibodies to cell surface antigens was found to result in either specific inhibition or enhancement of various stimulated cell functions. Immunoprecipitation and polyacrylamide gel electrophoresis were then used to identify the cell surface antigens recognized by these antibodies.

MATERIALS AND METHODS

Purification of human leukocytes. Human peripheral blood neutrophils and mononuclear cells were isolated from heparinized blood of healthy volunteers by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation.[9] Mononuclear cells were removed from the serum-gradient interface, while PMN were recovered from the cell pellet by dextran sedimentation and hypotonic lysis of contaminating erythrocytes. The resulting preparation contained 97% to 99% neutrophils and 1% to 3% mononuclear cells as assessed by microscopic inspections of Wright-stained smears. Viability was 95% to 97% by trypan blue exclusion. Peripheral blood eosinophils were purified by centrifugation of buffy coat cells over a discontinuous metrizamide gradient as previously described.[10] This technique yielded ≥90% eosinophils, with the remaining cells being PMN. Viability was >95%.

Mononuclear cells were further fractionated into adherent cells (90% monocytes, 10% lymphocytes) and nonadherent cells (97% lymphocytes, 3% monocytes) by incubation for one hour in plastic plates at 37 °C.[11] The nonadherent cells were fractionated into erythrocyte rosetting (E^+) and nonrosetting populations by incubation with sheep red blood cells and repeat centrifugation on Ficoll-Hypaque.[12] After RBCs were removed from the E^+ cells by lysis (in 0.85% ammonium chloride), the resultant population was found to be 95% lymphocytes with 97% viability. In studies of the specificity of antibody binding to different leukocyte subpopulations, "adherent cells" were used as monocytes and E^+ were used as lymphocytes.

Immunization and cell hybridization. Eight- to 10-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, Me) were immunized by intraperitoneal injection of 5×10^7 purified human neutrophils in complete Freund's adjuvant (Difco, Detroit). Initial immunization was followed by booster doses of 2.5×10^7 PMN in incomplete Freund's adjuvant at 14 and 28 days. One week later animals were bled and their sera screened for antineutrophil antibodies. Seropositive animals were injected intravenously with 1×10^7 human PMN; three days later their spleens were removed and single-cell suspensions were prepared.

Cell fusion was carried out according to the procedure of Kohler and Milstein.[13] Spleen cells (10^8) were fused with 2×10^7 P3NS-1 myeloma cells (ATCC #TIB18) in 35% polyethylene glycol (Sigma Chemical, St Louis) and 5% dimethyl sulfoxide (Fisher Chemical, Fairlawn, NJ) in RPMI 1640 (M.A. Bioproducts, Walkersville, Md).[14] Fused cells were incubated in RPMI containing 15% fetal calf serum (Hyclone, Logan, Utah) and hypoxanthine, aminopterin, and thymidine (HAT; reagents supplied by Sigma) at 37 °C in a humidified 5% CO_2 atmosphere. After 14 days the medium was changed to HT medium. Seven to 14 days later the supernatants from wells containing growing hybridoma cells were screened for the presence of antineutrophil antibody.

Screening of culture supernatants for monoclonal antibodies against PMN. Antineutrophil antibodies were detected by solid-phase radioimmunoassay using intact fixed cells. PMN (10^5) or other purified leukocytes were fixed to microtiter plates (Immulon

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Removawell Strips, Dynatech, Alexandria, Va) in the presence of glutaraldehyde (grade I, Sigma, kept at −20 °C until use) and 0.1% bovine serum albumin (RIA Grade, Sigma). Five percent rabbit serum in phosphate-buffered saline (PBS, pH 7.4) was then added to saturate nonspecific protein binding sites. The wells were extensively washed with PBS, and then 100 μL of hybridoma supernatant was added and incubated with the fixed cells for two hours at 37 °C. The plates were washed with PBS, and 50 μL of ^{125}I-labeled goat anti-mouse immunoglobulin were added (2×10^5 cpm; New England Nuclear, Boston). After incubation for two hours at 4 °C, the wells were washed three times with buffer, and the individual wells broken apart and counted. To determine the class and subclass of antibodies, immunodiffusion assays of hybridoma supernatants against antimouse IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 antisera (Research Products, Mount Prospect, Ill) were performed.[15]

Measurements of PMN function and effect of monoclonal antibodies. The effects of anti-PMN monoclonal antibodies on various cellular functions were examined in vitro using standard assays. These included (a) adherence to tissue culture plastic,[16] (b) PMN rosetting with IgG and IgM/complement-coated sheep red blood cells,[17] (c) PMN chemotaxis under agarose,[18] (d) superoxide generation by cytochrome C reduction assay,[19] and (e) PMN degranulation as measured by cell release of lysozyme and β-glucuronidase enzymes[20,21] with control for cell release of the cytoplasmic enzyme lactic dehydrogenase.[22] Cell stimuli included *N*-formyl-Met-Leu-Phe (FMLP; Sigma), phorbol myristate acetate (PMA; Sigma), zymosan-treated serum, and complement-opsonized zymosan.[1]

For these experiments, antibody-pretreated cells were prepared by incubating 10^7 PMN in 1 mL of endotoxin-screened (Pyrogent, Mallinkrodt, St Louis) hybridoma supernatant for ten to 30 minutes at 4 °C. Cells were then washed twice in Hanks' balanced salt solution without calcium or magnesium (HBSS, KC Biological, Lenexa, Kans) and added to complete medium (RPMI) or complete HBSS in experimental assays. Control experiments were performed using cells pretreated with P3NS1 (nonsecretory myeloma) supernatant or with supernatant containing irrelevant IgG monoclonal antibody.

For some cell function experiments, and for immunoprecipitation, purified monoclonal antibodies were prepared from malignant hybridoma ascites fluid obtained by injecting 10^8 hybridoma cells into the peritoneal cavities of BALB/c mice previously primed with 2, 6, 10, 14 tetramethyldecane.[23] Ascites fluid was harvested at 14 to 21 days, filtered, and chromatographed on DEAE affigel blue (Bio-Rad Labs, Richmond, Calif).[24] Fractions containing antibody, as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and radioimmunoassay (RIA), were pooled and IgG concentrated by lyophilization and 35% saturation ammonium sulfate precipitation. Purity was assessed by SDS-PAGE and immunodiffusion. $F(ab')_2$ fragments were prepared by pepsin digestion of purified IgG as previously described.[25]

Identification of PMN surface proteins detected by monoclonal antibodies. Cell surface proteins were labeled with ^{125}I by the iodogen (Pierce Chemical Co, Rockford, Ill) technique.[26,27] Purified PMNs (50×10^6) were washed twice in PBS and resuspended in 1 mL PBS. In some experiments, PMNs were then incubated in PBS containing 0.005 mol/L diisopropylfluorophosphate (DIFP, Sigma); after ten to 30 minutes' incubation with DIFP on ice, cells were washed and incubated 30 minutes in PBS before labeling to remove excess unreacted DIFP. Iodogen (10 μg) was dissolved in chloroform and dried onto the surface of a 20-mL glass scintillation vial. The vessel was first rinsed with PBS, and then the treated PMN were added along with 200 μCi carrier-free sodium ^{125}iodide (100 mCi/mL, New England Nuclear). After 30 minutes' incubation on ice, the cells were washed three times in cold PBS to remove unbound isotope and resuspended in 250 μL of extraction buffer (1% Triton X-100 [Fisher] containing 10 mmol/L iodoacetic acid/10 mmol/L phenylmethylsulphonylfluoride [Sigma]). Cells and detergent were vortexed every ten minutes, and after 30 minutes' incubation at 0 °C, the tubes were spun at 200 g for 20 minutes to remove nuclei and cellular debris.

In immunoprecipitation studies, 20 μg of ^{125}I-labeled cell extract protein was incubated with 200 μg purified monoclonal IgG (1-14, 1-15, or control). After two hours' incubation at 37 °C, 200 μg of the precipitating goat antimouse IgG (New England Nuclear) was added, and the antigen/antibody/antibody mixture was incubated overnight at 4 °C. The resulting precipitate was pelleted at 500 g for ten minutes at 4 °C and then washed three times with hypotonic buffer (0.1%, NP-40/0.1 mol/L NaCl/0.1 mol/L Tris, pH 7.4). The precipitated proteins were then reduced and denatured in buffer containing 5% 2-mercaptoethanol (Sigma) and 2% SDS (Bio-Rad) in 0.125 mol/L Tris, pH 6.8, and separated electrophoretically on an SDS-polyacrylamide slab gel. Radioactive bands on dried gels were identified by autoradiography.

Data analysis. Statistical significance of differences between experimental groups was determined by Student's *t* test for unpaired and paired data.

RESULTS

Production and screening of anti-PMN monoclonals. One hundred hybridoma clones of NS1 myeloma cells fused with splenocytes of PMN-immunized mice demonstrated regular growth and were screened for antineutrophil activity. Of these, 23 were positive by RIA against fixed human PMNs. Specificity of PMN recognition was determined by comparison of RIA-measured antibody binding to PMNs *v* other types of purified peripheral blood leukocytes. Clones 1-14 and 1-15 were selected for further study on the basis of apparent anti-PMN specificity (Table 1). By double immunodiffusion, antibody 1-14 was determined to be IgG_{2b}, while antibody 1-15 was found to be IgG_1. Subsequent PMN functional studies were performed using the culture supernatants produced from twice subcloned hybridoma cell lines.

Effects of monoclonal antibodies on superoxide generation and degranulation. Monoclonal antibody effects on PMN activation were studied initially by assay of PMN superoxide generation in response to 0.1 μmol/L FMLP and 10 ng/mL PMA (Table 2). After preexposure to Ab 1-14 containing supernatant, PMN release of O_2^- anion in response to FMLP was decreased by 64% (40 nmol/10^7

Table 1. Solid-Phase Radioimmunoassay of Monoclonal Antibody Binding to Purified Human Leukocytes

	Mean ^{125}I bound (cpm) to 10^5 Cells		
Purified Cell Population	Antibody 1-14	Antibody 1-15	Control IgG Monoclonal
Neutrophils	13,875*	15,960*	1,855
Eosinophils	3,124	4,354	2,345
Lymphocytes	3,059	3,254	2,292
Monocytes	2,200	3,200	2,215

Purified human leukocytes fixed to plastic wells were exposed to hybridoma supernatants containing monoclonal IgG. After incubation and removal of excess antibody by sequential washes, antibody binding was quantified by a second incubation with ^{125}I-$F(ab')_2$ fragments of sheep antibody against mouse IgG. Mean data are reported from three experiments.

*Significant difference from control IgG ($P < .05$) by paired *t* test.

Table 2. Effects of Monoclonal Antibodies 1-14 and 1-15 on PMN Degranulation and Superoxide Production in Response to FMLP and PMA

	PMN Preexposure	Degranulation* (% Total Cell Enzyme Released)		Stimulated O_2^- Production† (Net nmol O_2^- Released Over 10 min/10^7 PMN)
		Lysozyme	β-Glucuronidase	
Unstimulated PMN	Control IgG	3 ± 1	2.9 ± 0.3	Baseline
	Ab 1-14	6 ± 2‡ (+100%)	3.2 ± 0.6	None detected
	Ab 1-15	2 ± 1	2.0 ± 0.4	None detected
FMLP-Stimulated PMN (1 μM)	Control IgG	21 ± 2	3.8 ± 0.5	110 ± 40
	Ab 1-14	32 ± 3‡ (+52%)	5.0 ± 0.7	40 ± 20‡ (−64%)
	Ab 1-15	18 ± 4	2.7 ± 0.4‡ (−29%)	40 ± 20‡ (−64%)
PMA Stimulated PMN (10ng/ml)	Control IgG	4 ± 2	4.0 ± 0.6	220 ± 20
	Ab 1-14	11 ± 3‡ (175%)	6.0 ± 0.2‡ (+50%)	190 ± 10 (−14%)
	Ab 1-15	5 ± 2	2.7 ± 0.7	170 ± 20 (−23%)

*Mean ± SE granule release for five experiments assayed as enzyme content in cell supernatant after 30 minutes' incubation/enzyme content from 10^6 lysed PMN. Control for cell lysis was performed by measurement of the cytoplasmic enzyme lactate dehydrogenase (LDH). LDH release was ≤5% in all experiments.

†PMN release of superoxide anion measured as superoxide dismutase inhibitable reduction of cytochrome C (19) mean ± SE for five (PMA) or eight (FMLP) experiments.

‡Significant difference from response in control IgG ($P < .05$).

PMN/10 min *v* 110 nmol/10^7 PMN/10 min for PMNs preincubated in irrelevant monoclonal IgG, $P < .05$). Preincubation with Ab 1-15 likewise decreased PMN O_2^- response to FMLP by 64%. Furthermore, PMN superoxide release in response to PMA was reduced 14% by Ab 1-14 and 23% by Ab 1-15. Neither antibody produced inhibition of PMN superoxide release in response to zymosan-activated serum or to serum-opsonized zymosan (data not shown).

We next examined the effect of Ab 1-14 and Ab 1-15 on PMN degranulation response to FMLP and PMA. Incubation with monoclonal antibody 1-14 significantly increased degranulation in response to 1 μmol/L FMLP (Table 2); release of cell lysozyme was increased 52% ($P < .01$). Augmentation of PMA-induced degranulation also was noted (Table 2). In contrast, antibody 1-15 decreased cell granule release in response to FMLP, resulting in a 29% ($P < .05$) decrease in β-glucuronidase release. Viability assays and control assays for release of the cytoplasmic enzyme lactic dehydrogenase showed that the effects of antibodies 1-14 and 1-15 were not due to cytotoxicity. It was noted, however, that Ab 1-14 did significantly increase lysozyme release by unstimulated PMNs (6% of total cell enzyme release *v* 3% for control IgG ($P < .05$).

Effects of monoclonal antibodies on chemotaxis, adherence, and binding of opsonized particles. The ability of normal peripheral blood neutrophils to migrate in response to 0.1 μmol/L FMLP was diminished by monoclonal antibody 1-14. By contrast, incubation of PMNs in monoclonal 1-15 or control irrelevant IgG monoclonal had no effect on chemotaxis. In four separate experiments, the mean chemotactic index of cells incubated in the absence of antineutrophil antibody was 2.2 ± 0.2. Cells incubated in monoclonal 1-14 had a significantly lower chemotactic index (1.4 ± 0.1, $P < .02$) in response to FMLP. This decrease was found to be due to a specific 42% decrease in directed migration toward FMLP and not an effect on random cell migration.

Neutrophil adherence to plastic was significantly augmented by monoclonal antibody 1-14 under both unstimulated and FMLP-stimulated conditions. Preincubation of PMNs with antibody 1-14 increased unstimulated PMN adherence to tissue culture plastic by 115% (Table 3, $P < .05$) and FMLP-stimulated adherence by 111% ($P < .01$). In contrast, preincubation of cells with antibody 1-15 decreased unstimulated adherence by 64% ($P < .05$), and it also decreased FMLP-stimulated adherence (Table 3). There was no inhibition of PMA-stimulated cellular adherence in the presence of monoclonal 1-15.

In studies of cell adhesion to opsonized substrate, preincubation of PMNs in antibody 1-14 significantly reduced cell rosetting with IgM/complement-sensitized erythrocytes (74% decrease, $P < .01$; see Table 3). Ab 1-14 had no effect on cell rosetting with IgG-sensitized sheep erythrocytes, and preincubation with antibody 1-15 had no effect on PMN rosetting with either complement- or IgG-opsonized targets.

Effects of 1-14 and 1-15 purified IgG and F(ab')$_2$ fragments on PMN function. To determine the specificity of antibody effects on stimulated PMN function, further superoxide generation experiments were performed using highly purified 1-14 and 1-15 antibodies obtained from mouse hybridoma ascites. Alone, purified 1-14 and 1-15 IgG preparations were not stimulatory for PMNs. However, these purified antibodies, like the previously studied antibody-containing supernatants, produced significant inhibition of cell superoxide response to 0.1 μmol/L FMLP and 10 ng/mL PMA in a dose-dependent fashion: significant inhibition of PMN-induced superoxide generation was seen for IgG concentrations of 25 μg/mL (9% to 10% inhibition, $P < .05$) up to 500 μg/mL (60% to 80% inhibition, $P < .01$). F(ab')$_2$ fragments of 1-14 and 1-15 were also found to mediate significant inhibition of stimulated PMN superoxide production. In four separate experiments, 25 μg/mL of 1-15 F(ab')$_2$ reduced mean cell response to FMLP from 78 ± 11 nmol/10^7 cells/10 min [in the presence of control F(ab')$_2$] to 54 ± 12 nmol/10^7 cells/10 min, or by 34% ($P < .05$ by paired *t* test). Mean cell responses to PMA were reduced from 381 ± 22 (control) to 320 ± 36 nmol/10^7 cells/10 min, or by

Table 3. Effects of Monoclonal Antibodies 1-14 and 1-15 on PMN Adherence to Plastic and Binding of Opsonized Red Blood Cells

	PMN Preexposure	Adherence to Plastic (cells/well × 10^{-3})	Binding to IgSRBC	Binding to C'-SRBC (% rosetting PMN)
Unstimulated PMN	Control IgG	14 ± 1	76 ± 4	27 ± 4
	Ab 1-15	5 ± 1* (−64%)	82 ± 2 (+8%)	22 ± 4 (−19%)
	Control IgG	68 ± 24	76 ± 4	27 ± 4
	Ab 1-14	146 ± 41* (+115%)	78 ± 2 (+3%)	7 ± 1† (−74%)
FMLP-stimulated	Control IgG	67 ± 8	NT	NT
	Ab 1-15	39 ± 3* (−42%)		
	Control IgG	159 ± 38	NT	NT
	Ab 1-14	336 ± 68* (+111%)		

After ten minutes' preincubation with specific monoclonal or control supernatant, adherence to plastic was assayed by PMN incubation in tissue culture wells for one hour at 37 °C. Nonadherent cells were removed by gentle washing and adherent cells recovered and enumerated on a particle counter. Results reported as mean ± SE for three to seven assays. Because of interdonor variation in PMN adhesive response to plastic, mean control data for each experimental series are reported. To assay PMN binding of opsonized RBC purified human PMNs were incubated for 15 minutes at 37 °C with SRBC opsonized with either rabbit antisheep RBC IgG or rabbit antisheep RBC IgM/human serum. Rosetting was scored visually as >3RBC adherent/PMN, and results are reported as % rosetting PMN (mean ± SE) for three experiments. NT, not tested.

*Significant difference from PMN response with control IgG ($P < 0.05$), by Student's paired t test.

†$P < .01$.

16% ($P < .05$). In the same experiments, 25 μg/mL of 1-14 $F(ab')_2$ fragments also significantly reduced mean FMLP response to 57 ± 14 nmol/10^7 cells/10 min (27% decrease, $P < .01$) and PMA response to 333 ± 26 nmol/10^7 cells/10 min (13% decrease, $P < .02$).

Apparent molecular weights (mol wt) of the antigens recognized by monoclonals 1 to 14 and 1 to 15. The antigens detected by purified monoclonal antibodies 1-14 and 1-15 were characterized by immunoprecipitation of antigens from ^{125}I-labeled PMN membrane extracts. Proteins were separated by electrophoresis on reducing SDS-polyacrylamide gel. The results are shown in Fig 1. Antineutrophil antibody 1-14 recognized a surface protein of apparent mol wt 94,000. This band was not seen in precipitates formed with an irrelevant monoclonal IgG (control). Initial experiments indicated that the PMN antigen recognized by Ab 1-15 was variable in apparent mol wt (from 125,000 to 180,000); in later experiments, however, 30-minute pretreatment of PMNs with the protease inhibitor DIFP was found to result in reproducible Ab 1-15 immunoprecipitation of a molecule of 150,000 to 180,000 mol wt. Immunoprecipitation studies using either Ab 1-15 or Ab 1-14 with extracts of other peripheral blood cell types did not show ^{125}I-labeled protein precipitation. Using excess antibody, pre-precipitation of the 1-14 antigen from cell extracts did not remove the 1-15 antigen and vice versa.

DISCUSSION

In order to obtain specific reagents for use in structure-function analysis of the PMN surface, we screened newly developed anti-PMN monoclonal antibodies for their ability to modify PMN activation response in vitro. In initial study of hybridoma supernatants, we used a RIA against intact PMNs to increase chances of finding antibodies directed against PMN surface structures. After the anti-PMN specificity of these antibodies was established by RIA, their recognition of individual PMN surface structures was defined in immunoprecipitation studies. In assays of PMN function in vitro, two anti-PMN clones were found to produce antibodies that significantly altered cell function. Exposure to antibody 1-14 increased both PMN adherence and degranulation response in vitro but decreased directed migration of cells. Furthermore, Ab 1-14 reduced superoxide generation in response to FMLP and PMA and decreased the ability of PMN to rosette with complement-coated red cells, without affecting Fc-mediated binding or cell activation by complement-opsonized zymosan. In contrast, PMN exposure to antibody 1-15 reduced cell function in assays of adherence, chemotaxis, degranulation, and superoxide generation.

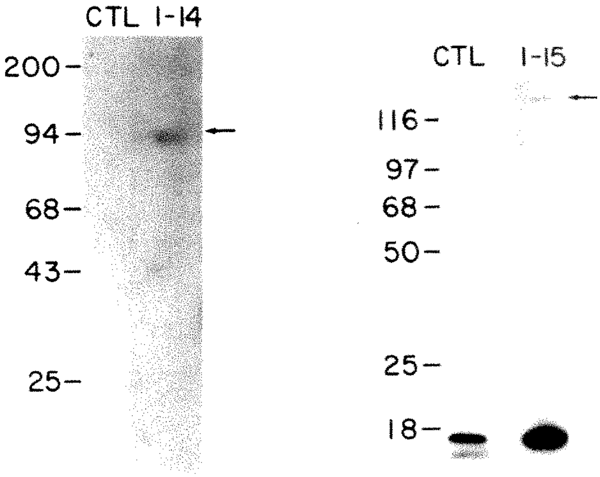

Fig 1. PMN antigen immunoprecipitation by monoclonal antibodies 1-14 and 1-15. Solubilized ^{125}I-labeled PMN surface proteins were incubated with Ab 1-15 or 1-14 for two hours at 37 °C and then precipitated with a goat antimouse IgG for 18 hours at 4 °C. Washed precipitates were reduced and denatured in 2-mercaptoethanol/SDS buffer and proteins separated on a 7.5% or 10% SDS-polyacrylamide slab gel. Autoradiography of the gel indicates specific recognition of a 150,000-mol wt molecule by antibody 1-15 (right-hand panel) and a 94,000-mol wt molecule by antibody 1-14 (left-hand panel). Lanes marked CTL indicate results for control monoclonal IgG in each experiment; migration of mol wt standards is indicated at the left of each panel.

Ab 1-15, however, had no effect on PMN binding of opsonized targets.

By these criteria, we developed two monoclonal cell lines that make antibodies with specificity for cell function-related neutrophil surface molecules of 94,000 and 150,000 to 180,000 mol wt. The individual antibodies, 1-14 and 1-15, had differing effects on PMN activation in response to FMLP and PMA; cell exposure to Ab 1-14 promoted cell activation in some assays and inhibited cell function in others. Exposure to Ab 1-15 either inhibited cell function or had no effect on cell response. Purified $F(ab')_2$ fragments of 1-14 and 1-15 were shown to mediate significant inhibition of PMN superoxide generation in response to FMLP and phorbol diester. This finding makes it unlikely that the function-modulating effects of 1-14 and 1-15 are due to cell activation or prestimulation by aggregating IgGs or to the specific effects of cell binding of the Fc portion of these IgG molecules. In our studies, we did not further investigate the possibility that isotype variations between 1-15 (an IgG_1) and 1-14 (an IgG_{2b}) outside the Fc region might account for their divergent function-altering effects. If isotype differences in cell recognition or binding are significant, our control IgG, an IgG_1, may not have been an appropriate control for antibody 1-14. Final resolution of the question of isotype influence awaits the development of new monoclonals of different isotypes that recognize the same antigen epitopes as antibodies 1-14 and 1-15.

Previous studies (Table 4) have also noted divergent affects of various monoclonal antibodies on PMN function in vitro. Cotter et al[28] and Beatty et al[29] have described antibodies, NCD 3 and 60.3, that inhibit PMN chemotaxis to FMLP. Martin et al[30] have developed a monoclonal that inhibits PMN oxidative metabolism induced by diverse stimuli. Anderson et al[8] mention unpublished data indicating that the monoclonal Mol inhibits PMN superoxide response to opsonized zymosan, and Painter et al[31] have also published preliminary data describing differential PMN inhibition and activation by three anti-PMN monoclonal antibodies. By contrast, Stevenson et al[32] have described two antineutrophil monoclonal antibodies that do not alter cell function. With the exception of Mol and 60.3, previous studies of antibody-mediated cell inhibition have not defined the surface antigens recognized by the monoclonal antibodies studied. This limits our ability to compare Ab 1-14 and 1-15 with these other antibodies.

The significance of membrane structures in PMN effector response to inflammatory stimuli has been evaluated by several groups.[33-39] Surface receptors have been described for the Fc portion of IgG (a 63,000/55,000 dimer[33]), for serum complement molecules (a 155,000 to 177,000 molecule with alpha and beta subunits for the C3bi receptor[34-36]), for FMLP (68,000 mol wt[37]), and for PMA.[38] To date, the C3bi receptor has been best studied because of its recognition by the monoclonal antibodies OKM1, Mol, Mac-1, and others.[8,20,36] Congenital absence of the Mol antigen has been associated with defects of C′ mediated binding as well as defects of PMN adherence, chemotaxis, aggregation, phagocytosis, and antibody-dependent cytotoxicity.[39] Because of its cell-activating effects, its inhibition of EAC rosetting, and its recognition of a 94,000-mol wt PMN surface structure, Ab 1-14 may be recognizing an epitope on or near the binding site of the 94,000-mol wt β-subunit of the C3bi complex. Along a similar vein, the monoclonal antibody 60.3 has been shown to recognize a 95,000/130,000/150,000 mol wt complex on neutrophils, lymphocytes, and monocytes, and it may be possible that antibodies 1-14 and 1-15 recognize subcomponents of this complex. When compared with the monoclonals OKM1/Mol and 60.3, however, it is worthy to note the high specificity of antibody 1-14 and antibody 1-15 for neutrophils. Because the C3bi receptor and the 60.3 antigen are known to be common to PMNs, monocytes, and lymphocytes, the apparent PMN specificity of Ab 1-14 and Ab 1-15 argues against their recognition of these molecules. Further, the function-modulating effects of 1-15 and 1-14 were not specific for either FMLP- or PMA-induced cell stimulation, and neither antibody had an effect on PMN superoxide response to opsonized zymosan. These data suggest that the 1-14 and 1-15 antibodies are not binding to specific receptor

Table 4. Summary: Alteration of PMN Function by Monoclonal Antibodies

			Reported Effects on PMN Function in Vitro			
Monoclonal Antibody	Adherence to Plastic	Binding of C′ Opsonized RBC	Chemotaxis	Metabolic Activation	Mol Wt of PMN Antigen	Reference
1-14	↑	↓	↓	↓O_2^- generation ↑Degranulation	94,000	—
1-15	↓	0	0	↓O_2^- generation ↓Degranulation	130,000 to 180,000	—
Mol	NP	↓	NP	↓O_2^- generation* ↓Degranulation	155,000/ 94,000	8,39
60.3	NP	NP	↓	NP	150,000 130,000 94,000	29
G2	NP	NP	NP	↓O_2 consumption	NP	30
NCB 3	NP	0	↓	0	NP	28
NMS 1	↓	NP	↓	↑Degranulation	NP	31
NMS 7	NP	NP	↓	↑Degranulation	NP	31
NMS 9	NP	NP	↑	0	NP	31

NP, not published; 0, no effect; ↑, increase; ↓, decrease.

*Reported as "unpublished data." Cell response to C′-opsonized targets.

molecules, but rather that they bind to molecules involved in the function of several PMN activation pathways. Further studies, including competitive binding and preabsorption experiments with purified antigens, will be required to define the specificity and cross-reactivity of OKM1, 60.3, 1-15, and 1-14. Immunoaffinity antigen purification should allow more precise definition of the 1-14 and 1-15 antigens and their role in the sequence of neutrophil activation.

Monoclonal antibodies have been shown to provide tools for study of the phenotypic expression of neutrophil antigens in developing granulocytes and myeloid tumor cells.[40-42] At present, the availability of nine PMN function-inhibiting monoclonal reagents offers researchers the potential to dissect specific pathways of PMN activation by inflammatory stimuli. Further studies using these reagents will allow isolation of individual PMN membrane molecules and identification of their function in neutrophil recognition of inflammatory stimuli.

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The Risks of Central Nervous System Relapse and Leukoencephalopathy in Patients Receiving Marrow Transplants for Acute Leukemia

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The records of 415 patients who received allogeneic marrow transplants for acute leukemia were reviewed to assess the risk of central nervous system (CNS) relapse and leukoencephalopathy after marrow transplantation. The Kaplan-Meier estimates of the probability of CNS relapse posttransplant were 13% for patients with acute lymphoblastic leukemia (ALL) and 2% for patients with acute nonlymphoblastic leukemia (ANL). Previous CNS disease was significantly correlated with an increased risk of CNS relapse in patients transplanted for ALL but not for ANL. In contrast, bone marrow involvement with leukemia at the time of transplant was associated with an increased risk of CNS relapse in patients with ANL but not in patients with ALL. Seventy-one patients with ALL did not receive posttransplant intrathecal methotrexate (IT-MTX) and 127 did. The probability of CNS relapse in these two groups was 38% and 7%, respectively ($P < .02$). This protective benefit from IT-MTX was present in patients both with and without a history of CNS involvement or marrow involvement at the time of transplant. In patients with ANL, 116 patients did not receive posttransplant IT-MTX and 101 patients did, but no protection from CNS relapse was observed from IT-MTX irrespective of a patient's previous CNS history or marrow status at the time of transplant. Leukoencephalopathy was seen exclusively in patients who had received radiation and/or intrathecal chemotherapy to the CNS before preparation for marrow transplantation and posttransplant IT-MTX. In such patients the risk of leukoencephalopathy was 7%. From our data, it appears that posttransplant IT-MTX is a significant benefit for ALL patients in preventing CNS relapse after marrow transplantation. A similar benefit from posttransplant IT-MTX for ANL patients cannot be established from this study. In both groups, increasing total CNS therapy was associated with an increasing risk of leukoencephalopathy.

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WE HAVE REPORTED the results for patients with acute leukemia who were transplanted in relapse after cyclophosphamide with or without other chemotherapeutic agents and total body irradiation (TBI).[1] In that study, four of 46 patients with acute lymphoblastic leukemia (ALL) relapsed initially in the central nervous system (CNS). In the 54 patients with acute nonlymphoblastic leukemia (ANL), there was one CNS relapse. It was anticipated that CNS relapses would increase as the fraction of early deaths and of patients dying of nonleukemic causes decreased in patients with ALL and possibly ANL. Based on these early studies, we initiated more intensive posttransplant prophylactic CNS therapy. This report presents an analysis of these attempts to eliminate CNS leukemia after transplant and to evaluate the occurrence of leukoencephalopathy.

MATERIALS AND METHODS

Four hundred fifteen patients with acute leukemia received allogeneic marrow transplants from December 1970 through December 1980 and were analyzed as of June 1984. In all patients, informed consent was obtained prior to transplantation. Protocols and consent forms were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. Patients transplanted at Children's Orthopedic Hospital have been reported elsewhere and are not included in the analysis.[2] Previous chemotherapy and CNS therapy varied with the disease history and the referring institution. All patients received TBI 9.2 to 10.0 Gy in a single exposure[3,4] or fractionated to a total dose of 12.0 to 17.5 Gy.[5,6] TBI was administered from opposing Cobalt 60 sources at a dose rate of 6 to 8 cGy/min. After TBI, 353 of the patients received cyclophosphamide, 120 mg/kg, in two divided doses. The remaining patients received a variety of supplemental chemotherapies as part of their conditioning regimen as described previously.[7] All patients received allogeneic transplants from family member donors who were HLA-compatible as determined by serologic typing and mixed leukocyte culture tests.[1]

The history of CNS leukemia was obtained from patient records. Active CNS disease was defined as the presence of leukemic blasts in the cytocentrifuge preparation or elevated and abnormal cerebrospinal fluid cell counts with clinical CNS symptoms at the time of initiating the marrow transplant preparative regimen.[8] Before 1976, patients received treatment for CNS disease on an individualized basis, and methotrexate (MTX) was administered intravenously (IV) on days 1, 3, 6, and 11 and then on a weekly basis until day 102 to prevent or ameliorate graft-*v*-host disease (GVHD).[1] From 1976 on, all patients were to receive two doses of intrathecal methotrexate (IT-MTX) in the ten days before marrow infusion (day 0). Posttransplant MTX was to be given intrathecally beginning on day 32 and on alternate weeks until day 102; IV-MTX was given on the alternate weeks. Patients with a history of or active CNS disease were to receive an additional eight doses of IT-MTX, given every other month after day 102. The IV-MTX dose was 15 mg/m^2 on day 1 and 10 mg/m^2 thereafter. The IT-MTX dose was 12 mg except in patients under 3 years of age, where 6 mg was used.[9]

After marrow transplantation, CNS relapse was defined either as meningeal leukemia occurring as the first site of recurrent leukemia or as meningeal leukemia occurring simultaneously with marrow recurrence. Patients who developed recurrent leukemia initially in marrow and then developed CNS leukemia were not considered to have had a primary CNS relapse.

Leukoencephalopathy (LEC) was defined as a clinical picture of lethargy, slurred speech, ataxia, seizures, confusion, dysphagia, spasticity, and decerebrate posturing with pathological findings of degeneration of the white matter with multifocal to confluent noninflammatory necrosis.[10]

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Tables 1 and 2 show the patient characteristics before transplantation. Patients were considered in remission if they had less than 5% blasts in the marrow. Of patients with ALL in remission, only 17 were in first remission; the rest were in a second or subsequent remission.

All estimates of the probability of CNS relapse and development of LEC were made using the method of Kaplan and Meier.[11] Differences between the patient groups were evaluated using the generalized Savage log rank test for censored observations.[12] Multivariate statistical methods were not used because of the low absolute frequency of CNS relapse and of LEC. Instead, the log rank test was used to rank potential risk factors and combinations of them. Risk factors evaluated included marrow status at the time of transplant, CNS leukemia involvement before or at the time of transplant, patient age, patient sex, acute or chronic GVHD, and TBI preparative regimen.

RESULTS

Central Nervous System Relapse

Acute lymphoblastic leukemia. Eleven of 198 patients transplanted for ALL relapsed in the CNS after marrow transplantation (Table 1). Six patients were in marrow remission and five patients were in marrow relapse at the time of transplant. Marrow status at the time of transplant was not associated with the risk of CNS relapse. Sixty-six of the 198 patients transplanted for ALL had a history of CNS involvement and/or CNS leukemia at the time of transplant, and they were found to have had a higher probability of CNS relapse after transplant than patients without a history of prior CNS involvement ($P < .02$).

Seventy-one of the 198 patients transplanted for ALL did not receive posttransplant IT-MTX. Forty-six did not receive IT-MTX because they were transplanted before the initiation of the posttransplant IT-MTX protocol, and 25 had IT-MTX omitted due to severe metabolic abnormalities, poor marrow graft function, or platelet support problems. Figure 1 shows the effect of posttransplant IT-MTX with respect to the probability of CNS relapse after transplant. There was a statistically significant decrease in the incidence of CNS relapse in patients who received posttransplant IT-MTX ($P < .02$). This improvement was seen independently of whether patients were in marrow remission or relapse at the time of transplant.

Table 1. Characteristics of Patients With Acute Lymphoblastic Leukemia

Bone Marrow Status at Transplant	Remission	Relapse
No. of patients	83	115
Age range in years (median)	3–30 (15)	2–49 (15)
History of and/or active CNS leukemia	28	38
CNS prophylaxis and/or treatment		
CNS irradiation		
No. of patients	58	74
Dose range (median)	1,600–3,900 cGy (2,400 cGy)	500–4,800 cGy (2,400 cGy)
Intrathecal methotrexate/ ARA-C		
No. of patients	72	73
No. with CNS relapse posttransplant	6	5
Time from transplant to CNS relapse, range in months (median)	2–27 (6/8)	1–17 (10)

Table 2. Characteristics of Patients With Acute Nonlymphoblastic Leukemia

Bone Marrow Status at Transplant	Remission	Relapse
No. of patients	115	102
Age range in years (median)	2–52 (25)	1–56 (23)
History of and/or active CNS leukemia	9	22
CNS irradiation		
No. of patients	9	14
Dose	1,800–2,400 cGy	1,500–2,400 cGy
Intrathecal methotrexate/ ARA-C		
No. of patients	13	15
No. with CNS relapse posttransplant	0	3
Time from transplant to CNS relapse, range in months (median)	—	1–5 (5)

When the effects of posttransplant IT-MTX were stratified according to the patient's CNS leukemia status at the time of transplant, it was found that there was a 19% probability of CNS relapse after transplant in patients without a history of CNS disease who did not receive IT-MTX, while only 4% of such patients who received IT-MTX relapsed. Among patients with a history of and/or active CNS disease, there was a 52% probability of CNS relapse in patients who did not receive IT-MTX, while the probability of CNS relapse was 17% among patients who did.

Other factors that failed to show an association with the risk of CNS relapse included acute or chronic GVHD, age, and sex.

Acute nonlymphoblastic leukemia. Three of 217 patients transplanted for ANL relapsed in the CNS (Table

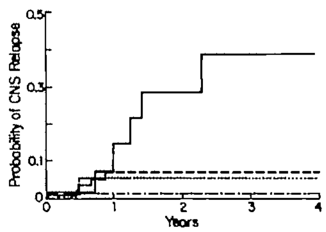

Fig 1. Kaplan-Meier probability of central nervous system relapse after marrow transplantation. Effect of posttransplant intrathecal methotrexate (IT-MTX) on patients with either ALL or ANL. (—) Patients with ALL who did not receive posttransplant IT-MTX (7/71 alive at day 1,460). (– – –) Patients with ALL who received posttransplant IT-MTX (29/127 alive at day 1,460). (· · · ·) Patients with ANL who did not receive IT-MTX (7/101 alive at day 1,460). (—·—·) Patients with ANL who received IT-MTX (45/116 alive at day 1,460). Patients with ALL who received IT-MTX had a significantly lower probability of CNS relapse than did patients with ALL who did not receive IT-MTX ($P < .02$). Differences in the probability of CNS relapse in patients with ANL grouped by posttransplant IT-MTX status were not statistically significant.

2). All three patients were in marrow relapse at the time of transplantation, and transplantation in marrow relapse was significantly associated with an increased risk of posttransplant CNS relapse ($P < .02$). Thirty-one of the 217 patients transplanted for ANL had a history of CNS involvement and/or CNS leukemia at the time of transplant. The influence of previous CNS disease was evaluated with respect to CNS relapse after transplant and no effect was detectable. There was a probability of 3% that patients with CNS disease before transplant would relapse in the CNS after transplant and a probability of 2% that patients who had not had previous CNS disease would relapse in the CNS.

Twenty-five patients transplanted in remission and 76 transplanted in relapse did not receive posttransplant IT-MTX. Two of the remission patients and 52 of the relapse patients were transplanted before the initiation of the IT-MTX protocol. Posttransplant IT-MTX was omitted in 47 patients due to severe platelet support problems, metabolic abnormalities, and/or poor marrow graft function. IT-MTX posttransplant did not significantly reduce the probability of CNS relapse (Fig 1). The slightly lower probability of CNS relapse in the group who received IT-MTX was entirely accounted for by the lower proportion of patients transplanted in marrow relapse in that group. Analysis of posttransplant IT-MTX in patients grouped by previous CNS disease status also failed to show any significant effect from receiving IT-MTX with respect to CNS relapse. Other factors with no detectable effect on the risk of CNS relapse included acute or chronic GVHD, age, and sex.

Leukoencephalopathy. Seven of the 415 patients developed LEC after marrow transplantation (Table 3). Due to the small numbers of patients who developed LEC, and because CNS toxicity should be independent of the type of leukemia, patients with ALL and ANL were evaluated together. There was no statistically significant relationship between a history of CNS disease and the development of LEC. All seven cases of LEC were found to have occurred among the 201 patients who had received either prophylactic or therapeutic treatment (radiation and/or IT therapy) of the CNS before marrow transplant. None of the 214 patients transplanted without a history of prior CNS treatment developed LEC ($P < .01$). All seven patients who developed LEC were in the group of 243 patients who received posttransplant IT-MTX ($P < .05$). Five of these seven patients were among the 57 patients who received six or more doses of IT-MTX posttransplant, one patient was among the 83 who received four to five IT-MTX doses, and one was among the 103 receiving one to three doses.

No patient who received posttransplant IT-MTX without previous CNS therapy developed LEC. Similarly, no patient who received previous CNS therapy without posttransplant IT-MTX developed LEC. Therefore, the risk of LEC was further evaluated only among patients who had previous CNS therapy and received posttransplant IT-MTX (Table 4). The overall risk of LEC in this group was 7%. Within this group, there was an increasing risk of LEC associated with an increasing number of doses of IT-MTX ($P < .01$).

Among the factors with no detectable effect on LEC were age, sex, acute or chronic GVHD, and bone marrow status at the time of transplant.

DISCUSSION

CNS prophylaxis has become routine for patients with ALL[13–15] but has been controversial in patients with

Table 3. Characteristics of Patients With Leukoencephalopathy

Unique Patient No.	635	764	1103	1176	1242	774	951
Patient No.	1	2	3	4	5	6	7
Diagnosis	ALL	ALL	ALL	ALL	ALL	ANL	ANL
Age at BMT	22	5	24	16	10	1.6	22
Prophylactic CNS irradiation							
Amount (cGy)	2,400	0	0	2,400	1,800	0	0
Years before BMT	2.3	—	—	2.5	2.5	—	—
Intrathecal prophylaxis							
Drug	MTX	—	—	MTX	MTX	—	Ara-C*
No. of doses	5	—	—	5	7	—	8
CNS relapses	—	1	2	1	—	1	—
CNS treatment							
Irradiation (cGy)	0	2,400	2,400	0	0	1,500	0
Years before BMT	—	0.16	1.2	—	—	0.66	—
Intrathecal therapy	—	Ara-C + MTX	MTX	Ara-C + MTX	—	MTX	—
Number	—	6	>12	6	—	10	—
CNS status at BMT	Neg	Neg	Pos	Neg	Neg	Pos	Neg
BMT preparation							
Total body irradiation (cGy)	1,000	1,500(f)	1,000	1,000	1,000	1,000	1,000
Doses IT-MTX	2	2	2	2	2	2	2
IT-MTX post-BMT							
No. of doses	6	6	6	1	6	6	4*
Onset of leukoencephalopathy (mo)	5	4	4.5	1	4.5	4	4

ALL, acute lymphoblastic leukemia; ANL, acute nonlymphoblastic leukemia; BMT, bone marrow transplant; CNS, central nervous system; MTX, methotrexate; Ara-C, cytosine arabinoside; f, fractionated; Neg, negative; Pos, positive; IT, intrathecal.

*IT therapy administered by way of Ommaya reservoir.

Table 4. Incidence of Leukoencephalopathy in Relationship to Presence or Absence of Pretransplant CNS Therapy and Extent of Posttransplant CNS Therapy.

No. of Doses of Posttransplant IT-MTX	Previous CNS Treatment*	
	Not†	Yes†
0	0/102	0/70
1-3	0/53	1/50
4-5	0/38	1/45
≥6	0/21	5/36

*Treatment defined as radiation to the CNS and/or IT chemotherapy as either CNS therapy or prophylaxis delivered before marrow transplantation.

†Number of patients with leukoencephalopathy/number of patients at risk.

ANL.[16-18] The purpose of this retrospective analysis is to define the risks of CNS relapse and the complications of CNS prophylactic therapy occurring after marrow transplantation. The data presented here were derived from two distinct time periods of marrow transplantation in Seattle. Among the first 100 patients, there were five relapses in the CNS, and the actuarial probability of CNS relapse for these patients was 21%.[19] In an effort to decrease this relapse probability, posttransplant IT prophylaxis with methotrexate was initiated. Six doses of posttransplant IT-MTX were selected for patients without a history of and/or active CNS leukemia at transplant, based on the assumption that a one $\log_{10}$ cell kill would result per dose of IT-MTX[20] and that the estimated number of blasts in occult leukemia is less than 10^6.[21]

The data presented here show that prophylactic CNS therapy posttransplant reduced the risk of CNS relapse for patients without a history of CNS leukemia who were transplanted for ALL. In patients without a history of CNS leukemia transplanted for ANL, no benefit of posttransplant IT-MTX could be demonstrated. However, the probability of CNS relapse for ANL patients given marrow grafts (2%) was lower than would be expected from the reported experience with patients treated with chemotherapy.[18] In fact, none of the 115 patients transplanted for ANL in remission relapsed in the CNS, and 39 of these patients are long-term survivors. This suggests that either our patient group is biased toward a population of patients at low risk for CNS relapse or, more likely, that the basic marrow conditioning regimen provides adequate CNS prophylaxis to this group.

Once CNS leukemia has occurred, the probability of achieving long-term continued complete remission is poor with conventional therapy.[22,23] Treatment of CNS leukemia is difficult because the number of intrathecal chemotherapeutic agents is small and drug resistance may develop rapidly. The irradiation therapy that the brain can safely receive is also limited.[24] Sixty-eight of 197 (35%) patients with ALL came to transplant with a history of and/or active CNS disease, and 31 of 218 (14%) patients with ANL came with CNS disease history or active CNS disease. The presence of CNS disease at any time was shown to be a significant risk factor for CNS relapse after marrow transplantation for patients with ALL ($P < .02$) but not for patients with ANL. However, patients with ALL and a history of CNS leukemia who received IT-MTX posttransplant experienced a threefold decrease in their incidence of CNS relapse, from 52% to 17%. In contrast, ANL patients with a previous history of CNS leukemia did not appear to benefit significantly from posttransplant IT-MTX with respect to CNS relapse.

As with most therapies, posttransplant IT-MTX was not without toxicity. Both irradiation and intrathecal chemotherapy have been associated with neurotoxicity, the most devastating of which is progressive LEC.[25-29] From our results, it appears that the risk of LEC after marrow transplantation is apparently not limited to children. Three of the seven patients in our study who developed LEC were over age 20 at the time of transplant, and age was not found to be a risk factor for the development of LEC in our analysis. In addition, LEC occurred only in patients who received pretransplant CNS therapy (CNS irradiation and/or IT chemotherapy) before the transplant. This suggests that neither the basic marrow conditioning regimen (consisting of cyclophosphamide, TBI, and IT-MTX) nor the addition of a course of posttransplant IT-MTX, is sufficient to cause a significant risk of LEC. However, most patients with ALL now routinely receive CNS prophylaxis.[15] Johnson et al[2] have reported that four of 24 marrow transplant recipients who had received CNS prophylaxis and who received six or more doses of IT-MTX posttransplant developed LEC (17%). In our study, we found that five of the seven patients who developed LEC were among 36 patients who received both CNS therapy before transplant and six doses of IT-MTX posttransplant (14%). Of the remaining two patients, one developed LEC after four doses of 12 mg MTX by way of Ommaya reservoir. Cerebrospinal fluid (CSF) MTX levels were measured and found to be markedly elevated. None of the other six patients who developed LEC had CSF MTX levels measured, and all received MTX by way of lumbar punctures. Lower doses of MTX administered by way of Ommaya reservoir may avoid neurotoxicity.[30,31] The remaining patient who developed LEC had received two full courses of IT therapy before marrow transplant and developed LEC after receiving only one dose of IT-MTX posttransplant.

As posttransplant IT-MTX appears to be beneficial in preventing CNS relapse in patients with ALL, we did attempt an analysis to determine the optimum number of IT-MTX doses after transplantation that would prevent CNS relapse. The data suggested that there was no added benefit from more than four to five doses of posttransplant IT-MTX irrespective of CNS leukemia status at transplantation. At these dosage levels, there is less than a 2% risk of developing LEC. More doses of IT-MTX were associated with an increased risk of LEC without any further protection from CNS relapse. It must be remembered, however, that these data were determined retrospectively, and that no uniform CNS treatment protocol was followed in our patients before transplantation. The optimum number of posttransplant IT-MTX doses is likely to vary from patient to patient and center to center, depending on the extent of previous CNS therapy and the perceived risk of CNS therapy.

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Disparities in Estimates of IgG Bound to Normal Platelets

By Neil Blumberg, Debra Masel, and Mark Stoler

Estimates of the number of IgG molecules bound to normal platelets have ranged from several hundred to several tens of thousands. The lower estimates were generated from direct binding assays and stoichiometric assumptions. The higher values derive from competitive binding assays, in which platelet-associated IgG (PAIgG) is calculated from a standard curve using soluble IgG standards. Using a kinetic-ELISA (enzyme-linked immunosorbent assay) antiglobulin assay, we measured normal platelet IgG to be 21,200 ± 9,400 molecules per platelet when a competitive assay and soluble IgG standards were used. Direct measurement of bound antiglobulin by kinetic-ELISA and stoichiometric assumptions yielded a measurement of 259 ± 117 IgG molecules per platelet. Soluble IgG and PAIgG are not comparable in their ability to bind anti-IgG. Disparities in estimates of normal PAIgG are probably due to methodological differences. The estimate most likely to be correct is several hundred IgG or less per normal platelet.

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ESTIMATES OF NORMAL LEVELS of platelet-associated IgG (PAIgG) have generally ranged from 1 to 10 fg per platelet.[1,2] This corresponds to 4,000 to 40,000 molecules of IgG per platelet, or 10^2- to 10^3-fold greater than estimates for normal red cell-associated IgG.[3] Two reports have estimated normal PAIgG levels at 169 and 235 molecules of IgG per normal platelet, respectively.[4,5] LoBuglio et al[4] suggested their lower results might be due to their use of a monoclonal anti-IgG reagent and to elimination of platelet fragments from their assay.

In general, all studies with higher estimates of PAIgG used competitive assays. In these, platelet binding of anti-IgG is compared with the binding of standard concentrations of soluble IgG to anti-IgG. The two lower estimates were derived from calculations of the theoretical maximum number of anti-IgG molecules present in the antiglobulin reagent, followed by direct measurement of the proportion of anti-IgG binding to normal platelets.[4,5] The high levels of PAIgG reported in most studies have raised questions as to the specificity of the assays used to measure PAIgG.[6-8]

Ware et al[9,10] have reported that use of an assay calibrated with soluble IgG as standards gave greatly increased estimates of PAIgG compared with the estimates derived from a direct monoclonal anti-IgG binding assay and stoichiometric calculations. They reported that using a direct binding assay, monoclonal anti-IgG studies yielded a result of 500 to 1,000 IgG molecules per normal platelet.[10] Using polyclonal anti-IgG, the result was 2,000 to 3,000 IgG per cell.[10] To confirm these findings and evaluate methodological variations as a cause for disparities in estimates of normal levels of PAIgG, we measured PAIgG by two methods, using the same polyclonal antiglobulin reagent in each. One method was a competitive binding assay using calibration by soluble IgG standards. The other method used measurement of anti-IgG binding to normal platelets and stoichiometric derivation of the number of anti-IgG molecules bound.

MATERIALS AND METHODS

Competitive assay for PAIgG using calibration with a soluble IgG standard curve. In this assay, PAIgG or soluble IgG standards compete with solid-phase IgG for binding to an alkaline phosphatase-linked anti-IgG antiglobulin reagent. The principle of the assay is that increased concentrations of soluble IgG or PAIgG will compete for anti-IgG and reduce anti-IgG binding to the solid-phase IgG. The solid-phase is a 96-well microtiter plate (Immulon II, Dynatech, Inc, Alexandria, Va) that has been previously washed three times with distilled water. All washes are performed by machine (Miniwash, Dynatech). A 100-μL solution of 100 ng purified human IgG (Pelfreeze Biologicals, Rogers, Ark) in coating buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, pH 9.8) is placed in each well and incubated overnight at 4 °C. Two wells are coated and incubated with 100 ng of purified bovine serum albumin (BSA) (Sigma Chemical Co, St Louis) in coating buffer as negative controls. No binding of anti-IgG–alkaline phosphatase conjugate should occur in these two wells.

Platelets are prepared from EDTA anticoagulated normal donor blood collected in evacuated collection tubes (Sherwood, St Louis). Platelet-rich plasma is made by centrifugation at 250 g for ten minutes, and three washes are performed with phosphate-buffered saline (PBS)-EDTA (3.0 g/L Na_2 EDTA, 3.75 g/L Na_2HPO_4, 8.2 g/L NaCl, pH 7.0). Platelets are resuspended at a count of 50,000/μL in PBS-Tween (PBS as earlier, without EDTA, but with 0.5 ml/L Tween 20 [Sigma, St Louis]). Removal of possible platelet fragments was not attempted.

A series of dilutions of purified IgG is prepared containing 25, 50, 100, 250, 500, 750, and 1,000 ng in 100 μL of PBS-Tween. These serve as soluble standards to compete with the solid-phase IgG for alkaline phosphatase–anti-IgG conjugate.

The microtiter plate coated with IgG is washed four times with PBS-Tween before use. The soluble IgG standard curve dilutions are added to individual wells in triplicate, as are 100 μL of 50,000/μL solutions of washed platelets (total 5 × 10^6 per well). Several duplicate wells are used as negative controls. The BSA-coated wells receive 100 μL of PBS-Tween. Two IgG-coated wells receive 100 μL of PBS-Tween to serve as zero points for competitive binding (ie, maximal binding of anti-IgG due to lack of competing soluble IgG). Two wells coated with IgG receive neither soluble IgG, platelets, nor anti-IgG, to serve as controls for spontaneous substrate conversion. PBS-Tween (200 μL) is added instead. Once all soluble IgG standards and the platelets have been added, 100 μL of a 1:300 dilution (in PBS-1% BSA) of alkaline phosphatase coupled to affinity-purified antihuman IgG (Sigma) is added to each well, except as noted earlier. The plate is then gently rotated (Microshaker, Dynatech) for one minute, covered with a plastic seal, and incubated at 37 °C for one hour. The contents are then dispelled by inversion, and the plate washed four times with PBS-Tween. Substrate is added to each well (200 μL of 1 mg/mL *p*-nitrophenylphosphate (Sigma) in 5.3 g/L Na_2CO_3, 0.2 g/L $MgCl_2 \cdot 6H_2O$, pH 9.8).

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The plate is rotated for 18 minutes, and determination of bound anti-IgG conjugate is performed automatically by a through-the-plate spectrophotometer (Dynatech MR600) coupled to an Apple IIe computer (software program available from Dr Jonathan Cowles, 149 Tobey Rd, Pittsford, NY 14534). Bound anti-IgG conjugate is determined kinetically as ΔA_{405nm}/min × 1,000. Bound anti-IgG has been shown to be linearly proportional to this slope in a kinetic-ELISA (enzyme-linked immunosorbent assay).[11] A standard curve of slope (ΔA_{405nm}/min × 1,000) *v* ng IgG in the soluble standards is constructed to calibrate the assay. This is linear over the range of the assay when a log-linear plot is used (see Fig 1). The quantity of IgG bound to 5×10^6 platelets is calculated by using the measured mean ΔA_{405nm}/min × 1,000 and the standard curve. Values are reported as 10^{-15} g (fg) IgG per platelet. These are converted to molecules of IgG per platelet assuming a molecular weight (mol wt) of 150,000 daltons.

Direct binding assay for PAIgG. The alternative method for measuring PAIgG involves stoichiometric calculation of the activity of the alkaline phosphatase–anti-IgG conjugate (number of anti-IgG molecules added per test) and measurement of the proportion of the conjugate that actually binds to 5×10^6 platelets. The calculations of the number of anti-IgG added are shown in the next section.

The measurement of percentage binding to 5×10^6 platelets is performed as follows: The microtiter plate is not coated with IgG, since it serves only as a reaction vessel. Washed platelets (5×10^6) (100 μL) are incubated with 100 μL of a 1:300 dilution of the same alkaline phosphatase–anti-IgG conjugate used in the competitive assay. The incubation conditions are identical: 37 °C for one hour. The platelets are sedimented at 500 g for ten minutes and washed at 500 g for five minutes four times with PBS-Tween. Loss of platelets under these conditions is usually <10%. Substrate is added and ΔA_{405nm}/min × 1,000 is determined exactly as in the competitive assay. Here we measure anti-IgG conjugate bound to platelets rather than to the solid-phase IgG on the plate. The amount of alkaline phosphatase–anti-IgG added is quantitated by measuring the ΔA_{405nm}/min × 1,000 of the unreacted original anti-IgG conjugate, before its incubation with platelets. Thus, a ratio of ΔA_{405nm}/min × 1,000, platelets per original anti-IgG conjugate is a measure of the proportion of the added anti-IgG conjugate that has been bound to the platelets. By calculating (activity bound to platelets) × (number of anti-IgG conjugate molecules added) we arrive at an estimate of the maximum number of anti-IgG molecules present on the platelets.

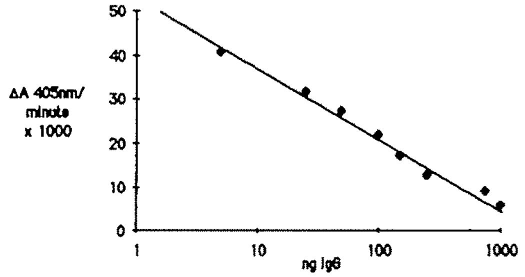

Fig 1. Typical scatter diagram for the competitive kinetic-ELISA. Standard dilutions of purified IgG are plotted on the logarithmic axis *v* the mean ΔA_{405nm}/min × 1,000 measured on the linear axis. Increasing amounts of soluble IgG reduce the binding of anti-IgG–alkaline phosphatase conjugate to the solid-phase IgG. A linear standard curve is constructed by least squares linear regression of ΔA_{405nm}/min × 1,000 *v* the log of the IgG concentration. The *r* value for this line usually is 0.99 or better. The nanograms of IgG per 5×10^6 platelets in the assay is derived by interpolation on this standard curve using the mean measured ΔA_{405nm}/min × 1,000 for the platelet suspension.

Calculation of the maximum number of anti-IgG molecules present in the alkaline phosphatase–anti-IgG conjugate. A number of assumptions must be made to allow these calculations: (1) enzyme and anti-IgG biological activity are unchanged by the conjugation process; (2) the ratio of anti-IgG to enzyme in the conjugate is 1:1; and (3) each anti-IgG–enzyme conjugate binds to one IgG on the platelet surface. The overall effect of these assumptions is likely to be overestimation of the number of binding sites on the platelet, since (1) the method used for conjugation probably leads to <1% of the conjugate being fully immunologically and enzymatically reactive[12]; (2) the ratio of anti-IgG to enzyme in the conjugate is probably much less than one, since threefold more enzyme than anti-IgG by weight is reacted in the conjugation process[13]; and (3) the third assumption, if incorrect, could underestimate PAIgG by twofold, if all anti-IgG–enzyme conjugate molecules were bound to two IgG molecules each, the theoretical maximum.

The calculations are as follows:

1. Conjugate enzyme activity, as measured by the manufacturer: 208 U/mL. Units added per well: (208 U/mL) (0.1 mL) (1/300 dilution) = 6.93×10^{-2} units.
2. Specific activity of the alkaline phosphatase before conjugation: 1,000 U/mg = 0.001 mg/U.
3. Mass of enzyme added per well = (6.93×10^{-2} units) (0.001 mg/U) = 6.93×10^{-5} mg.
4. Mol wt of alkaline phosphatase ≈100,000.
5. Moles of enzyme added per well = (6.93×10^{-5} mg) (10^{-8} mol/mg) = 6.93×10^{-13} mol.
6. Assume 1:1 conjugation of enzyme to anti-IgG. Thus moles of enzyme = moles of anti-IgG.
7. (6.93×10^{-13} mol anti-IgG) (6.02×10^{23} molecules per mole) = 4.17×10^{11} molecules of anti-IgG per reaction.
8. Maximum number of anti-IgG reacted per platelet is thus, theoretically:

$$\frac{4.17 \times 10^{11}\ \text{anti-IgG}}{5 \times 10^{6}\ \text{platelets}} = 83{,}400 \text{ molecules of anti-IgG per platelet.}$$

RESULTS

The mean normal PAIgG using the competitive assay, calibrated by standard curves of soluble IgG to convert ΔA_{405nm}/min × 1,000 to mass units, was 5.3 ± 2.36 fg per platelet (n = 324) (±1 SD). Using a mol wt of 150,000 daltons for IgG, this converts to approximately 21,300 IgG molecules per platelet. PAIgG of patients with immune thrombocytopenia are usually fivefold to 100-fold higher than these levels (Fig 2). Patients with acute leukemia and sepsis also have elevated levels of PAIgG in many instances.

The proportion of conjugate binding to normal platelets in the direct assay, as quantitated by the ratio ΔA_{405nm} platelets/ΔA_{405nm} unreacted conjugate, was 0.31% ± 0.14% (n = 5). That this low-level binding is not due to inadequate amounts of anti-IgG in the 1:300 dilution of antiglobulin conjugate is evidenced by the fact that binding increases many fold when platelets heavily coated with IgG are used in the assay as described. Conversion to number of IgG molecules per platelet is by multiplying the mean percentage binding (0.31%) by the maximum theoretical number of anti-IgG molecules reacted per platelet (83,400) = 259 molecules per platelet.

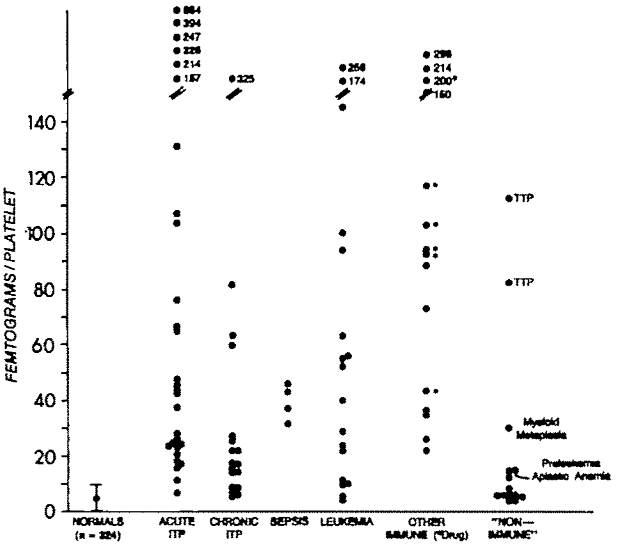

Fig 2. Pattern of elevated PAIgG in selected patients with various types of thrombocytopenia. ITP, idiopathic thrombocytopenic purpura; TTP, thrombotic thrombocytopenic purpura. Further details on these patients are available from the authors. Determinations by competitive assay employing soluble IgG standards.

DISCUSSION

Our results confirm the findings of Rosse et al[9,10] that disparities between high and low estimates of normal PAIgG levels are the result of differences in methodology and calculation technique. We believe that the estimates based on stoichiometric calculations and direct measurement of anti-IgG binding, such as those used by Lepoirrier's,[5] LoBuglio's,[4] and Rosse's[9,10] groups, are likely to represent truer estimates of PAIgG. They rest on scientifically reasonable assumptions and are not likely to be incorrect by more than a factor of two. As discussed in Materials and Methods, the assumptions used are, in fact, more likely to overestimate PAIgG. Although most PAIgG assays use a two-stage antiglobulin procedure,[1] we used a typical competitive one-stage immunoassay.[14] There are no theoretical or practical reasons why this should have affected our results.

Methods for PAIgG determinations calibrated using soluble IgG rest on the assumption that PAIgG and soluble IgG standards compete equally well for anti-IgG. Ware et al's[9,10] data argue strongly against the accuracy of this assumption. One possible explanation for this disparity is that the location on the platelet of PAIgG might favor bivalent binding kinetics for an anti-IgG reagent, rather than the univalent binding kinetics that apply to solution reactions between anti-IgG and IgG. The difference between univalent and bivalent affinity constants can be as much as tenfold to 100-fold.[15] This possibility could be studied by using anti-IgG reagents with univalent binding properties [eg, F(ab) fragments].

In summary, use of a standard curve of soluble IgG to measure levels of PAIgG overestimate the actual number of molecules per platelet by approximately 80-fold in our hands. Results of PAIgG estimates using soluble IgG standards should perhaps be reported as "fg per platelet in soluble IgG equivalents." We believe that our data and those in the literature are most consistent with a maximal estimate of <300 molecules of IgG per normal platelet.

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Human Thrombocytopenia Is Associated With Structural Abnormalities of the Endothelium That Are Ameliorated by Glucocorticosteriod Administration

By Craig S. Kitchens and J.F. Pendergast

Capillary fragility is characteristic of severe thrombocytopenia. This mechanical weakness may not be solely accounted for by decreased ability of platelets to repair endothelial breaks. Platelets may have a role in maintaining endothelial hemeostasis. This laboratory has demonstrated thinnning of capillary endothelium in experimental thrombocytopenia. We now report similar findings in human thrombocytopenia. Capillary endothelium supplying either skin or skeletal muscle was found to have a mean thickness only half that of normal as well as frequent very thinned areas, including some fenestrations. All findings reverted toward normal after four days of prednisone administration at a time the degree of thrombocytopenia was equally severe. These findings are consistent with the hypothesis that platelets are necessary for normal structure and function of endothelial cells and that glucocorticosteroid administration may ameliorate the pathophysiology of thrombocytopenia.

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ADEQUATE NUMBERS of functional platelets are necessary for normal hemostasis. Platelets seal endothelial breaks in the event of rupture. Claiming that lack of this sealing function alone does not account for capillary fragility seen in severe thrombocytopenia, it has been hypothesized that platelets additionally, in some manner, may play a role in maintaining endothelial integrity.[1,2] This laboratory has published morphological data in experimental thrombocytopenia in rabbits supporting this hypothesis.[3] We have additionally demonstrated that endothelial structural alterations in this experimental model were ameliorated by the administration of glucocorticosterioids.[4] Herein are reported observations made on tissue obtained from humans with severe thrombocytopenia.

MATERIALS AND METHODS

Patients. Strict criteria for patient accession accounts for the few patients accrued over a five-year period. Patients had to have severe thrombocytopenia ($\leq 15{,}000/\mu L$) not due to a process (chemotherapy or radiation therapy) that conceivably could directly damage a variety of tissues, including endothelium. The platelet count had to remain $\leq 15{,}000/\mu L$ after four days of daily administration of prednisone, 1 mg/kg, in order to minimize endothelial structural changes related primarily to resolution of thrombocytopenia. Before their entry into the study, they could not have received glucocorticosteroids. Because of our strong feelings that glucocorticosteroids are effective for hemorrhage associated with severe thrombocytopenia, we could not ethically accept transfer of thrombocytopenic patients from other cities without first recommending the administration of such agents. This greatly restricted the number of patients who were available to serve in this study comparing determinations made before and during prednisone administration. Finally, patients had to agree to serve in the experiment, all parts of which were approved by the Internal Review Board of the University of Florida.

Tissue. As rapidly as possible after initial evaluation and understanding of the protocol, biopsies were performed after which prednisone, 1 mg/kg/d, was begun. Biopsy material was also obtained on the fourth day of such treatment if the platelet count was still $\leq 15{,}000/\mu L$. If the platelet count was $\geq 15{,}000/\mu L$, the original biopsy material was not further processed. Biopsy material was obtained from skin and muscle. Skin biopsies were taken from the leg in areas near fresh petechiae but not of petechiae themselves. This area was chosen to ensure that capillary fragility was present. Our previous animal studies demonstrated that capillary thinning was not confined to actively hemorrhaging areas, being just as pronounced in normal-appearing tissue.[3,4] An elliptical incision 6 × 2 mm was made with a surgical scalpel deep enough to reach subcutaneous tissue. The wound was closed with three sutures, and pressure dressings were applied. Tissue from the gluteal muscle was obtained using a Lee soft-tissue biopsy needle (Becton Dickinson, Atlanta), which was placed roughly along the axis of the indicated bone marrow aspirate and biopsy tract. Biopsy yielded muscle in both pretreatment and posttreatment attempts in only three patients; therefore, muscle data are complete for only three patients. Data regarding skin biopsy is complete for all five patients. Tissues were then immediately diced in cold 3% glutaraldehyde in phosphate buffer and postfixed in OsO_4. It was then embedded, sectioned, and stained with lead citrate and uranyl acetate as previously described.[3]

Electron microscopy. All material was examined using a Philips 300 electron microscope (Eindhoven, The Netherlands). All photomicrographs were made at a fixed magnification (50,000) without knowledge of patient or status of prednisone administration. It was impossible to be unaware of whether the biopsy source was skin or muscle. Several blocks from each patient sample were examined until approximately 100 capillaries from each biopsy sample had been photographed. All vessels encountered were photographed, regardless of photogenicity, in order to minimize bias in choosing which vessels to photograph.

Collection of data. Before breaking the patient and preparation code, each photomicrograph was examined for (1) "thin spots," (2) fenestrations, and (3) mean thickness of the capillary endothelium. Figure 1 demonstrates these terms. A thin spot is defined as an area in which the endothelium attenuates to a thickness of only 700 to 800 A, the thickness of an endothelial vesicle. This degree of thickness is admittedly arbitrarily selected but being markedly thinner than normal endothelium (4,000 to 6,000 Å), such areas were readily identifiable. Fenestrations are not found in normal endothelium of capillaries supplying muscle or skin (except capillaries supplying sweat glands).[5] Mean capillary thickness was determined using planometric methods previously described.[4] In our experience, this method has a variability of ±2.7%. Because tissue from these patients before they developed thrombocytopenia was not available,

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The work was done when Dr Kitchens was a Clinical Investigator of the Veterans Administration.

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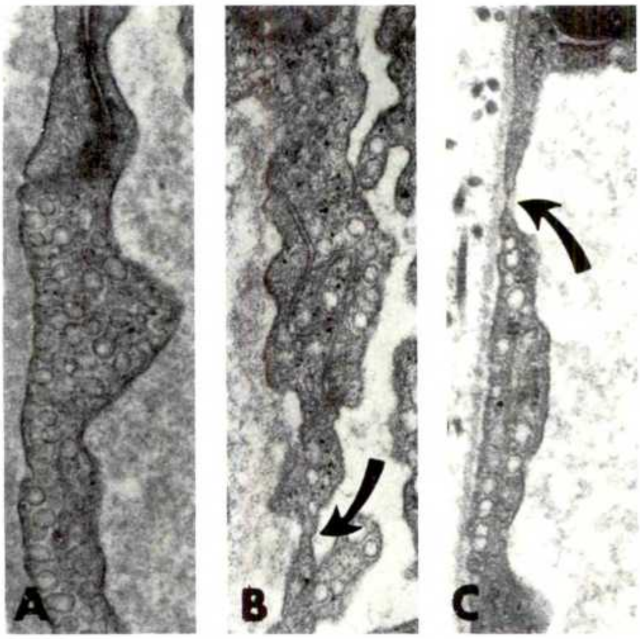

Fig 1. Definition of morphological terms. (A) demonstrates normal endothelium with a thickness of 3,000 to 5,000 Å. (B) shows attenuation to a point of 700 A and is referred to as a "thin spot" (arrow). A fenestration is shown in (C). All figures oriented with capillary lumen to the right and all ×33,000.

determinations from other studies this laboratory has performed using human skin and muscle capillaries were used to define normal.[6,7] The methods were identical. To our knowledge, glucocorticosteroid administration does not affect normal mammalian capillary endothelial structure.[4,6-8] The data were analyzed using the Statistical Analysis System (SAS)[9] computer software package on an IBM 4341 machine in the Faculty Support Center at the University of Florida. Significance of changes in capillary thickness measures was determined using a one-sided paired t test on measures weighted to adjust for the variability within subjects. Comparisons between the study group and normal tissue were made using a two-sample t test, again after weighting the measures to adjust for within-subject variability.[10]

RESULTS

Five patients fulfilled all criteria and their results form the basis of this study (Table 1). Three were diagnosed as having immune thrombocytopenia purpura (ITP) resistant to prednisone administration. Two were initially diagnosed as having amegakaryocytic thrombocytopenia[11,12]; one subsequently developed aplastic anemia, whereas the other's disease evolved into acute nonlymphocytic leukemia.

The response of each patient to prednisone administration was noteworthy. Whereas each patient manifested fresh petechial formation and epistaxis on admission, such hemorrhagic phenomena resolved with the commencement of glucocorticosteroid administration, although the patient's thrombocytopenia did not substantially improve. Likewise, the initial biopsy sites bled significantly, whereas those made on the fourth day bled only little more than similar biopsies performed on patients having normal platelet counts.[13] These events correlated with their template bleeding times, all of which were >20 minutes before prednisone administration and all of which were <20 minutes (mean, 16.5 ± 2.2 minutes; normal, <9.0 minutes) during prednisone administration.

Table 1. Patient Characteristics

			Platelet Count/$10^3/\mu L$	
Age	Sex	Diagnosis	Base Line	1 mg/kg/d Prednisone × Four d
58	F	AMT	4	4
73	M	AMT	3	3
18	F	ITP	2	10
88	M	ITP	2	6
71	M	ITP	11	15

AMT, amegakaryocytic thrombocytopenia; ITP, immune thrombocytopenic purpura.

Table 2 gives our morphological data. Data from the five patients (five pairs of skin biopsies and three pairs of muscle biopsies) were pooled for statistical analysis. Despite the small number of subjects, the capillary endothelium of both skin and muscle is thinner ($P = .001$ and $P = .013$, respectively) and shows more thin spots and fenestrations than normal human endothelium. Additionally, these changes tend to revert toward normal after the administration of glucocorticosteroids ($P = .001$ for skin and $P = .055$ for muscle) at a time when the patients' bleeding was clinically markedly abating. Skin and muscle capillary endothelium in thrombocytopenia after prednisone administration is still thinner than normal endothelium ($P = .001$ for skin and $P = .008$ for muscle). All interendothelial junctions observed were normal.

Data from each individual were also analyzed. Mean capillary thickness was thinner ($P < .05$) in all three muscle and five skin biopsies during thrombocytopenia compared with normal. Mean capillary thickness increased significantly ($P < .05$) after glucocorticosteroid administration in two of the three muscle samples and in all five of the skin specimens. There was no difference in the data whether the thrombocytopenia was due to decreased production (amegakaryocytic thrombocytopenia) or increased destruction (immune thrombocytopenia).

DISCUSSION

The results of this study are remarkably similar to those obtained in our studies of experimental thrombocytopenia

Table 2. Observation of Human Microvascular Endothelium

Material	Thickness + SD	Thin Spots*	Fenestrations*
Skin: normal (n = 36)	5,881 ± 2,399 Å	0.0	0.0
Skin: thrombocytopenia (n = 120)	2,832 ± 933 Å	43.3	6.7
Skin: thrombocytopenia and prednisone (n = 100)	4,040 ± 1,702 Å	5.0	1.0
Muscle: normal (n = 100)	4,180 ± 1,321 Å	0.0	0.0
Muscle: thrombocytopenia (n = 109)	2,074 ± 737 Å	44.9	5.5
Muscle: thrombocytopenia and prednisone (n = 147)	2,552 ± 1,013 Å	20.4	1.3

*Per 100 capillaries.

produced in the rabbit.[4] In each situation, the endothelium during thrombocytopenia becomes roughly half the original thickness, and thin spots and fenestrations are encountered in 43% and 6%, respectively, of the capillaries encountered. After prednisone administration, the endothelial thickness reverts toward normal, assuming approximately 75% of original thickness, while thin spots and fenestrations largely, but not totally, disappeared. Using scanning electron microscopy, we demonstrated enfacement of the capillary endothelial surface in experimental thrombocytopenia. These changes also were partially reversed by prednisone administration.[14] Endothelial alterations similar to those found in humans were also found in dogs rendered severely thrombocytopenic by infusion of thrombin.[8]

As these results during thrombocytopenia are consistent with decreased mechanical strength of the capillary endothelium[7] at a time when capillary fragility and hemorrhage are manifest, it is attractive to hypothesize a casual relationship. As both structural alterations and bleeding are ameliorated by glucocorticosteroid administration, this hypothesis is further strengthened.

The mechanism by which either platelets or glucocorticosteroids may support normal endothelial structure, and hence strength, is not known at this time, but several possibilities exist. Endothelium could appear thinner if fewer endothelial cells were available to cover a fixed luminal surface area. It is unlikely that the number of endothelial cells changes as rapidly as necessary to explain the changes observed in either this clinical study or the previous experimental studies.

Endothelium could appear thinner if the mean circumference of the microcirculation increased. We do not observe any differences among groups of patients (or animals in experimental work) with respect to diameter of capillaries or to how many capillaries were "open" or "closed" as determined by relaxation or constriction of arterioles proximal to the microcirculation. Thinned or fenestrated endothelium was just as likely to be found in a small, flat (ie, "closed") capillary as in a nearly perfectly round capillary. As capillaries themselves lack smooth-muscle cell investiture, their "openness" is determined by proximal blood flow. The law of LaPlace determines that the thin-walled capillary, because of its overall small diameter compared with the rest of the vascular tree, is most resistant to passive engorgement or overinflation due to proximal pressure.

Most likely these alterations are caused by the metabolic effects that platelets and glucocorticosteroids have on endothelial cells. These effects are imperfectly understood and are now being discovered. Platelets can provide prostaglandin endoperoxides to endothelial cells for metabolism.[15,16] King and Buchwald[17] have demonstrated a factor in platelets that promotes endothelial cell growth and is distinct from platelet-derived growth factor. Blajchman et al[18] have demonstrated that hydrocortisone administration decreases vessel wall prostacylin production in vivo. Profound alterations in protein synthesis by endothelial cells studies in vitro are induced by glucocorticosteroids.[19] Any or all of these observations may in part play a role in endothelial cell homeostasis.

Shepro et al[20] were unable to find similar capillary ultrastructural changes in nonquantified observations in experimental thrombocytopenia. The wide variation in endothelial thickness observed in the present study indicates that many vessels in thrombocytopenic preparations may appear normal and many capillaries in normal rabbits or humans may appear thinner than the mean. Only by observing and quantifying many vessels in a blinded morphometric analysis will such changes be statistically apparent.

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Platelet Glycoproteins IIb and IIIa Associated With Blood Monocytes Are Derived From Platelets*

By Richard B. Levene and Enrique M. Rabellino

Platelet glycoprotein IIb/IIIa (GP IIb/IIIa), the receptor complex for fibrinogen, has been regarded as a megakaryocyte/platelet lineage-restricted antigen. Recently, however, it has been reported that GP IIb/IIIa is expressed in blood monocytes. Studies were performed to establish the origin and immunological characteristics of monocyte-associated glycoproteins IIb and IIIa (GPs IIb and IIIa). Preparations of blood monocytes containing varying platelet-monocyte ratios were metabolically labeled with [^{35}S]methionine with the expectation that any newly synthesized GPs IIb and IIIa would be monocyte-derived, since platelets have only rudimentary protein synthetic apparatuses. Analyses of sodium dodecyl sulfate (SDS) gels of homogenates of cell preparations containing from 200 to 5:1 platelet-monocyte ratios revealed that unlabeled GPs IIb and IIIa were readily immunoisolated using protein A-Sepharose immunobeads. However, fluorographic analyses of the same cell preparations pulse-labeled with [^{35}S]methionine failed to demonstrate synthesis of GP IIb or IIIa. Additionally, no GP IIb or IIIa was detected when immunoisolation was carried out in pure preparations of monocytes containing <1:100 platelet-monocyte ratios and SDS acrylamide gels were stained by the sensitive silver stain method. Furthermore, heterologous polyspecific antisera and two monoclonal antibody preparations against GPs IIb and IIIa, which bound to platelets, failed to bind to monocyte membranes. Thus, evidence was presented that indicated that monocytes do not synthesize platelet GPs IIb and IIIa and that detection of these molecules in blood monocyte preparations reflects platelet contamination.

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PLATELET GLYCOPROTEINS IIb and IIIa (GPs IIb and IIIa) are major constituents of the platelet plasma membrane and form a complex (GP IIb/IIIa) that participates both in the binding of fibrinogen and in platelet aggregation.[1-3] GPs IIb and IIIa are significantly diminished or absent in patients with Glanzmann's thrombasthenia, a hereditary bleeding disorder characterized by defective platelet aggregation and fibrinogen binding.[4-6] Platelet GPs IIb and IIIa are synthesized by megakaryocytes and expressed since early stages of megakaryocytopoiesis.[7-11] Virtually all mature megakaryocytes, as well as early immature megakaryocytes (promegakaryoblasts) and the clonable megakaryocyte progenitors, bear these antigens.[7-10,12] In addition, recent reports suggest that monocytes may also express platelet GPs IIb and IIIa.[13-17] In this communication, studies were carried out to characterize the platelet GPs IIb and IIIa associated with blood monocytes and to determine whether monocytes synthesize these platelet glycoproteins. No monocyte synthesis of GPs IIb and IIIa was detectable. It was also shown that detection of GP IIb/IIIa in monocyte preparations was due to platelet contamination.

MATERIALS AND METHODS

Preparation of human mononuclear blood cells and platelets. Venous blood was collected from normal volunteers in 0.32% sodium citrate or 5 mmol/L EDTA (final concentration) in polypropylene test tubes. For experiments requiring full platelet depletion, EDTA anticoagulated blood was diluted in equal volumes of 0.3 mmol/L EDTA calcium- and magnesium-free Hanks' balanced salt solution (GIBCO, Grand Island, NY) containing 1.25×10^{-5} mol/L sodium citrate, 2.52×10^{-5} mol/L HEPES buffer, and 4.1×10^{-6} mol/L sodium bicarbonate at pH 7.0 (HBSS/C) and centrifuged at 1,135 g for 15 minutes at 4 °C. The buffy coat was collected and processed by sequential differential centrifugations at 4 °C.[18,19] Mononuclear cell preparations were obtained from leukocyte concentrates depleted of erythrocytes and platelets by buoyant density centrifugation on gradients generated with solutions of colloidal silica particles coated with polyvinyl pyrollidone (Percoll, Pharmacia Fine Chemicals, Piscataway, NJ) in HBSS/C containing 0.3 mmol/L EDTA at 22 °C. Cells harvested from the interface of the gradient layers (d < 1.077 g/cm^3) were subsequently processed through four to five cycles of incubations with autologous serum containing 5 mmol/L EDTA and intervening differential centrifugations, as described by Pawlowski et al.[20] Platelet contamination was evaluated by microscopic examination of cell suspensions and Wright-Giemsa–stained smears.

For experiments in which the testing populations contained various platelet-nucleated cell ratios, buffy coats and platelet-rich plasma were generated from citrated venous blood and processed independently. In these experiments, gradients were designed to maximize monocyte enrichment, and cells were resuspended directly in gradient solutions. After centrifugation, low-density mononuclear cells of buoyant densities <1.077 g/cm^3 were collected from the upper medium layer and the gradient interface. Cells from the gradient layer (d = 1.077 g/cm^3), which were primarily lymphocytes, were selectively excluded. Washed platelets were obtained from platelet-rich plasma as described elsewhere.[7] Platelets were resuspended in Tris–HCl–citrate buffer at pH 6.4 containing 63 mmol/L Tris, 95 mmol/L NaCl, 5 mmol/L KCl, and 12 mmol/L citric acid and stored at 37 °C in polypropylene tubes. Before reconstitution, platelet counts were performed with a Coulter counter (Model ZBI; Coulter Electronics, Hialeah, Fla).

Gel-filtered platelets were prepared by passage of citrated platelet-rich plasma through Sepharose 2B equilibrated in HEPES-

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*While this manuscript was undergoing revision, a communication by Clemetson et al has reported the absence of platelet membrane glycoproteins IIb/IIIa from monocytes as analyzed by two-dimensional gel electrophoresis, immunoprecipitation, and crossed immunoelectrophoresis. (Clemetson KJ, McGregor JL, McEver RP, Jacques IV, Bainton DF, Domzig W, Baggiolini M: Absence of platelet membrane glycoproteins IIb/IIIa from monocytes. J Exp Med 161:972, 1985.)

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buffered Tyrode's solution containing 3.8 mmol/L HEPES, 3.8 mmol/L NaH_2PO_4, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.2% BSA, and 0.1% dextrose at pH 7.35, as described elsewhere.[19]

Antisera. Monospecific rabbit antisera against purified human platelet GPs Ib, IIb, and IIIa, and polyspecific rabbit antiplatelet membrane antisera were prepared and tested for specificity as described previously.[21-23] Monoclonal antibody PC-1 with specific reactivity to the platelet GP IIb/IIIa complex was generated and screened as described elsewhere.[12] Polyspecific rabbit antimouse Fab Ig was a generous gift from Dr Howard Grey (National Jewish Hospital, Denver). IgG fractions of all rabbit and mouse antisera were prepared by 50% saturated ammonium sulfate precipitation, DEAE-cellulose chromatography, and, subsequently, conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) (BBL Microbiology Systems, Walkersville, Md).[7] The conjugated proteins were fractionated by column chromatography on DEAE-cellulose, using stepwise elution with sodium phosphate buffers, pH 7.4, of 0.01, 0.05, and 0.1 mol/L. In general, 0.05 mol/L sodium phosphate buffer eluted antibody preparations with a dye-protein molar ratio of 1 ± 0.3 and was found to have the highest specific staining activity. Additionally, a monoclonal antibody against platelet GP Ib was kindly provided by Dr Barry Coller (SUNY, Stonybrook, NY).[24] Monoclonal antibodies B1, T3/T11, and Mol, with specific reactivities against B lymphocytes, T lymphocytes, and monocytes, respectively, were purchased from Coulter Immunology (Hialeah, Fla). Rabbit IgM antimouse IgG used to enhance complement fixation in the cytolytic assay was prepared by short-term immunization and subsequent precipitation and chromatography as described previously.[12]

Radioiodination of membrane proteins. Cell surface proteins on mononuclear blood cells and washed platelets were radioiodinated with ^{125}I by the tetrachloro-diphenylglycoluril method (Iodo-Gen, Pierce Chemical Co, Rockford, Ill).[25] Briefly, 5×10^6 mononuclear cells or 2×10^9 platelets resuspended in 200 μL phosphate-buffered saline (PBS) were incubated with 100 μCi of ^{125}I in the presence of 250 μg 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodo-Gen) for ten minutes at 22 °C. Tubes were precoated with 250 μg of Iodo-Gen dissolved in 125 μL chloroform under gentle stream of nitrogen. After iodination, unbound ^{125}I was removed by washing cells three times with PBS, and cells were solubilized as described later.

Metabolic labeling of mononuclear cells and platelets. Aliquots of isolated blood mononuclear cells (40×10^6) or platelets (1×10^9) resuspended in 1 mL methionine-free minimum essential medium (MEM)/Eagle supplemented with 0.015 mol/L L-glutamine, 5% whole fetal calf serum (FCS), and 10% methionine-free FCS were pulse-labeled with 400 μCi [^{35}S]methionine (1,100 Ci/mmol) at 37 °C for four hours. FCS was heat-inactivated at 56 °C for 30 minutes. Methionine-free FCS was prepared by extensive dialysis against methionine-free MEM. Unbound label was removed by washing cells, and homogenates of cell proteins were generated by solubilizing cells with 1% Triton X-100 in the presence of 2 mmol/L phenlymethylsulfonylfluoride, 5 mmol/L *N*-ethylmaleimide, 10 mmol/L EDTA (all from Sigma Chemical Co, St Louis) and 1 μmol/L pepstatin (Protein Foundation, Osaka, Japan). Validation of endogenous incorporation of radioactive amino acids into neosynthesized protein was obtained by pusle-labeling cells in the presence of increasing concentrations of the protein synthesis inhibitor cycloheximide as well as by evaluating radioactive label incorporation into TCA-precipitable protein.

Immunoprecipitation, SDS gel electrophoresis, and fluorography. Homogenates of mononuclear cells and platelets were subjected to solid-phase immunoadsorption with protein A-Sepharose CL-4B beads (Pharmacia) coated with antibodies monospecific for platelet GPs IIb and IIIa after extensive preclearing. Binding efficiency of antibody to protein A-Sepharose CL-4B beads was between 17 and 19 μg/μL beads. Binding efficiency of a radioiodinated antibody preparation was estimated by measuring radioactivity bound to washed beads over specific labeling activity expressed in cpm/μg protein. The efficiency of the immunoprecipitation system used was estimated by measuring removal of GP IIb/IIIa from a radioiodinated platelet membrane preparation. Assessment of antigen precipitation was conducted on platelet lysates by sequential adsorptions with antibody-coated beads. In a representative experiment, 255 μg antibody coupled to 15 μL beads removed all GP IIb/IIIa derived from a lysate of 2×10^9 platelets detectable by autoradiography. Preclearing was performed by sequential incubation of lysates with immunoadsorbant beads and beads coated with normal rabbit or mouse IgG. After incubation of cell homogenates for four to six hours at 4 °C with rotation, immunobeads were washed three times with 20 mmol/L Tris-HCl containing 500 mmol/L NaCl and 1% ovalbumin (pH 7.8) and three times with 20 mmol/L Tris-HCl containing 150 mmol/L NaCl (pH 7.8). Subsequently, immobilized protein was eluted by incubation of beads in 20 mmol/L Tris-HCl containing 2% SDS and 6 mol/L urea (pH 6.8) for five minutes at 100 °C. Eluants were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing and nonreducing conditions.[26,27] Gels were subsequently examined for immunoisolated proteins after processing for fluorography, silver stain, or Coomassie blue staining with estimated levels of sensitivity of approximately 1 to 10 pg, 1 to 5 ng, and 0.5 to 1 μg protein, respectively.[27-30]

Immunofluorescence assays, flow cytometry, and cell sorting. For fluorescence staining of membrane-restricted components, isolated cells were incubated with 20 to 50 μL of various antibody preparations in the presence of PBS containing 0.02% sodium azide (NaN_3) and 2% bovine serum albumin (BSA). After incubation at 4 °C for 30 minutes, cells were washed twice in PBS–BSA–NaN_3 and examined with Ploem illuminator and phase-contrast optics or processed by flow cytometry. To block functionally the Fc region of IgG molecules, antibody preparations were pretreated with *Staphylococcus aureus* protein A at wt-wt ratios of 7:1. $F(ab')_2$ fragments of second antibodies were also used. For fluorescence staining, smears of the various types of cell preparations were fixed with pure methanol at room temperature for 15 minutes, washed with PBS three times, and incubated with the different antibody preparations in a wet chamber at 20 °C for 30 minutes. After washing twice in PBS, cells were examined by ultraviolet microscopy. Cells were double-assayed for two antigens by staining cell preparations simultaneously or sequentially with a TRITC-conjugated antiserum or an FITC-conjugated specific reagent.[8]

Flow cytometric analysis was performed with a Coulter EPICS V (Coulter Electronics) equipped with Coherent 94-S argon ion laser. Cells in suspension were excited with laser adjusted to 1 W at 488 nm for FITC and 514 nm for TRITC at a flow rate of approximately 1,500 to 3,200 cells per second using a 76-μ flow tip. Sorting of cell subpopulations was performed using both green fluorescence and forward angle light scatter after defining appropriate electronic windows. To assess the effectiveness of the sorting procedure, positively sorted cells were reanalyzed by flow cytometry and phase-contrast microscopy for expression of Mo-1 antigen and latex particle phagocytosis, respectively. Data were analyzed with a Coulter EASY data-processing computer.

Complement-mediated cytolysis. Preparations of mononuclear cells depleted of most free-floating platelets were washed twice in McCoy's 5-A medium. Aliquots of 3×10^6 cells were incubated with 375 μL of monospecific antisera or ammonium sulfate precipitated mouse monoclonal antibody preparations at various concentrations for 30 minutes at room temperature and, subsequently, washed twice in McCoy's 5-A medium containing 10% heat-inactivated fetal bovine serum. Antibody-treated cells were then resuspended in 375 μL undiluted oxidized normal human AB Rh_D+ serum that was prepared by iodination and used as a source of complement, and incubated at 37 °C for 30 minutes.[31] To enhance complement fixation by monoclonal antibodies, antibody-treated cells were incubated with a rabbit IgM antimouse IgG at room temperature for 30 minutes and washed twice before complement treatment. Cell viability was assessed by supravital staining with trypan blue and observation under phase-contrast microscopy.

RESULTS

Immunoisolation of platelet glycoproteins IIb and IIIa from low-density human mononuclear blood cell preparations. The presence of platelet GPs IIb and IIIa in monocytes was investigated in preparations of low-density mononuclear cells generated after depletion of free-floating platelets. Typically, the mononuclear cell preparations contained approximately 56% monocytes and 44% lymphocytes. Cell differential analyses were performed by immunofluorescence using monoclonal antibodies Mol, B1, and T11/T3 specific for monocytes and B and T lymphocytes, respectively. Platelet-monocyte ratios, reflecting platelet contamination, were determined by examination of cell suspensions under phase-contrast microscopy and differential analyses of Wright-Giemsa–stained smears. Mononuclear cell homogenates were subjected to immunoisolation using protein A-Sepharose beads coated with rabbit antibodies monospecific for GPs IIb and IIIa. Two bands of approximately 126,000 and 116,000 daltons, corresponding to the molecular weights of reduced platelet GPs IIb and IIIa, were clearly identified in SDS gels of reduced immunoisolated material obtained from a cell preparation of 20 × 10^6 mononuclear cells containing 11 × 10^6 monocytes and 3.26 × 10^8 platelets (platelet-monocyte ratio = 30) (Fig 1B). Visualization of the nonreduced monomeric GPs IIb and IIIa molecules was precluded by the heavily stained band of the antibodies' IgG molecules (Fig 1A). Parallel controls using normal rabbit IgG are depicted in Fig 1C and D.

The presence of platelet GPs IIb and IIIa in a cell preparation of 40 × 10^6 mononuclear cells containing 20 × 10^6 monocytes and 2.86 × 10^6 platelets (platelet-monocyte ratio = 0.14) was also investigated in immunoisolated material eluted from beads coated with rabbit antiplatelet GPs IIb and IIIa after radioiodinating cell membrane proteins by the chloroglycoluril method. Autoradiograms of immunoisolated material electrophoresed in 7.5% acrylamide SDS gels revealed trace amounts of GPs IIb and IIIa, which comigrated with clearly identifiable GPs IIb and IIIa isolated in parallel from platelets (data not shown).

Assessment of synthesis of platelet GPs IIb and IIIa by blood monocytes. To assess whether platelet GPs IIb and IIIa immunoisolated from low-density blood cells were platelet- or monocyte-derived, studies were conducted to investigate the biosynthesis of these proteins. These studies were undertaken with the expectation that platelets, unlike monocytes, have only rudimentary protein synthetic apparatuses, and that any newly synthesized protein would be monocyte-derived. In a series of experiments, monocyte preparations containing varying degrees of platelet contamination were metabolically labeled with [^{35}S]methionine and analyzed for the synthesis of GPs IIb and IIIa. Material immunoisolated using antiplatelet GP IIb and IIIa antisera was electrophoresed in 7.5% SDS gels and analyzed concurrently by fluorography and Coomassie blue staining. Immunoprecipitates isolated from a preparation of 80 × 10^6 mononuclear cells containing 40 × 10^6 monocytes and 8 × 10^9 platelets (platelet-monocyte ratio = 200) were analyzed under nonreducing and reducing conditions (Fig 2). Although two bands of 125,000 and 116,000 daltons were readily recognized in the reduced Coomassie blue stained gel (Fig 2B), no comparable proteins were observed in either the nonreduced or the reduced counterpart fluorograms (Fig 2A′ and B′).

In another experiment, a preparation of 80 × 10^6 mononuclear cells containing 40 × 10^6 monocytes and 1.48 × 10^8 platelets (platelet-monocyte ratio = 5) was studied in a similar manner and neither GP IIb nor IIIa was detected (data not shown). Material derived from the same immunoprecipitation when analyzed in gels loaded with ninefold increased amount of protein revealed that although a trace of protein was visualized in Coomassie blue stained gels (Fig 2D), no detectable neosynthesized GP IIb or IIIa was observed (Fig 2C′ and D′). The specificity of the metabolic labeling system of monocytes was established by pulsing cells in the presence of increasing concentrations of cycloheximide (Fig 3). Cycloheximide at a concentration of 1 mmol/L fully inhibited protein synthesis of 2 × 10^6 cells.

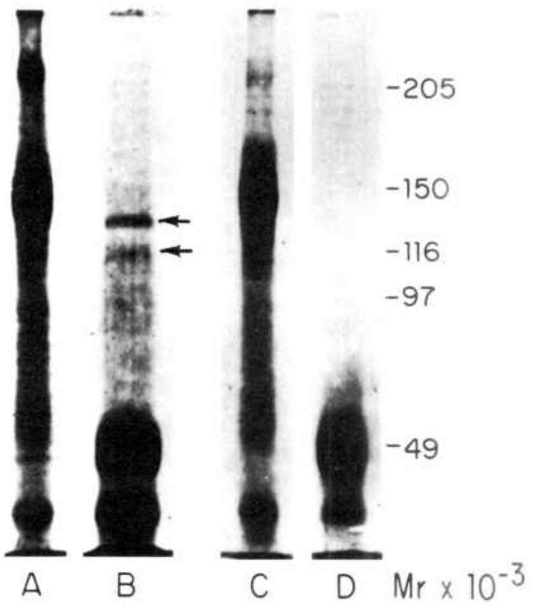

Fig 1. Electrophoretic analysis of platelet GPs IIb and IIIa immunoisolated from a mononuclear blood cell lysate solubilized in 5 mmol/L EDTA, 1% Triton X-100, 20 mmol/L Tris-HCl at pH 6.8 (nonreduced A, reduced B) in 7.5% acrylamide SDS gel stained with Coomassie blue. Arrows indicate immunoisolated GPs IIb and IIIa. Cell preparation contained 11 × 10^6 monocytes and 3.26 × 10^8 platelets (platelet-monocyte ratio = 30). Gels shown in C and D correspond to material eluted from control beads coated with normal rabbit IgG and electrophoresed under non-reducing and reducing conditions, respectively. Molecular markers are indicated at the right: myosin, 205,000 daltons; immunoglobulin G, 150,000; β-galactosidase, 116,000; phosphorylase B, 97,000; egg albumin, 49,000.

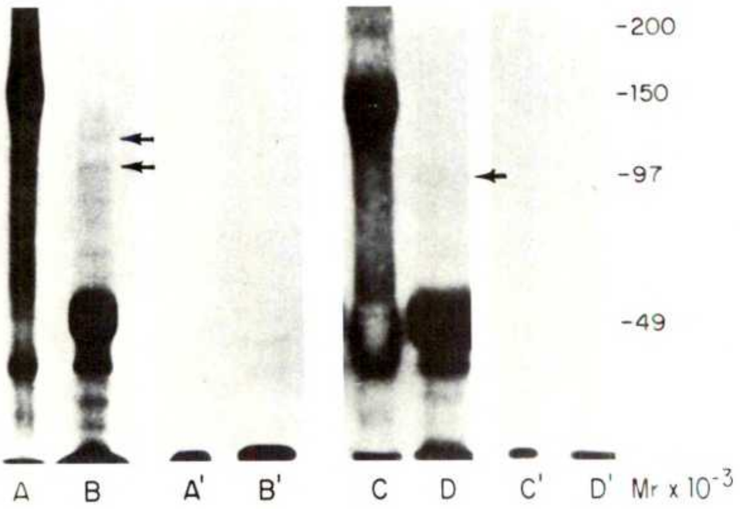

Fig 2. Eluants of [^{35}S]methionine-labeled mononuclear homogenates (platelet-monocyte ratio = 200) extracted from immunobeads coated with rabbit antiplatelet GPs IIb and IIIa analyzed concurrently by Coomassie blue staining (non-reduced A, reduced B) and fluorography (nonreduced A, reduced B) after 7.5% SDS-PAGE. Similar immunoisolated material obtained from preparations containing platelet-monocyte ratio = 5 (C, C' non-reduced, D, D' reduced). Arrows indicate immunoisolated GPs IIb and IIIa. Molecular markers are indicated on the right: myosin, 200,000 daltons; immunoglobulin G, 150,000; phosphorylase B, 97,000; egg albumin, 49,000.

GPs IIb and IIIa were studied in a highly pure preparation of monocytes obtained by flow cytometric sorting. Blood monocytes depleted of free-floating and most adherent platelets were stained using a fluorescent-labeled monoclonal antibody specific for a human monocyte cell surface antigen (mol wt 90/150 kd; Mol, the CR_3, or complement receptor type three) expressed on monocytes and absent in the majority of lymphoid cells (except natural killer cells) and platelets (Coulter Immunology).[32] Figure 4 shows the flow cytometric distribution of the mononuclear cell preparation as a function of the light-scattering and fluorescent intensity. The boxed image corresponds to selected sort windows that includes Mol-bearing monocytes. Flow cytometry provided a preparation of 3.36×10^6 monocytes, representing more than 98% nucleated cells, that contained 3.3×10^4 platelets (platelet-monocyte ratio = 1:100). The presence of GPs IIb and IIIa in sorted monocyte populations was investigated by SDS-PAGE of immunoisolated material. Electrophoretic analysis revealed that no platelet GPIIb or IIIa were detected in the cell preparation despite the fact that gels were stained by the sensitive silver stain method (data not shown).

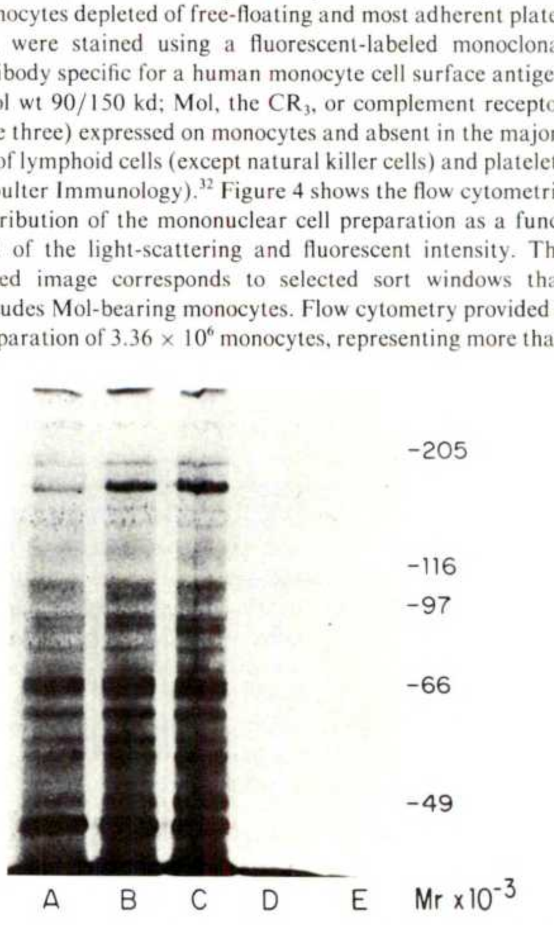

Fig 3. Fluorogram of protein homogenates obtained from mononuclear blood cells pulse-labeled with [^{35}S]methionine after electrophoresis in 7.5% acrylamide SDS gel under nonreducing conditions (A). (B), (C), (D), and (E) correspond to profile of radiolabeled proteins from cells pulsed in the presence of 1, 10, 100, and 1,000 μmol/L cycloheximide, respectively. Molecular markers are indicated on the right: myosin, 205,000 daltons; β-galactosidase, 116,000; phosphorylase B, 97,000; bovine serum albumin, 66,000; egg albumin, 49,000.

In order to exclude the synthesis of GPs IIb and IIIa by platelets, preparations of fully purified platelets were metabolically labeled and subjected to immunoadsorption in a similar manner. However, minimal specific labeling was detected corresponding to 3.9×10^{-5} TCA precipitable dpm/platelet, as compared with monocytes, which incorporated 1.805 dpm/cell when pulsed under the same conditions. Furthermore, increasing concentrations of cycloheximide resulted in inhibition of [^{35}S]methionine incorporation. More important, no neosynthesis of GP IIb or IIIa was detected when platelet homogenates were subjected to immunoprecipitation.

Identification of cells bearing platelet GPs IIb and IIIa in mononuclear cell preparations. The expression of platelet GPs IIb and IIIa on the membrane and in the intracellular compartment of isolated human blood cells was investigated by immunofluorescence using heterologous and monoclonal antibody preparations. For staining of membrane-restricted

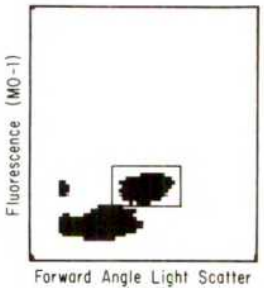

Fig 4. Two-dimensional flow cytometric distribution of mononuclear blood cells analyzed by forward angle light scatter (x) and binding of fluorescent-labeled Mol antibody (y). Boxed image indicates selected sort windows.

proteins, preparations of viable cells were incubated with specific antibody preparations. Staining of intracellular antigens was achieved by incubating cell preparations with antibody preparations after smearing and fixation, which rendered the membranes permeable to the antibody molecules. In mononuclear cell preparations obtained from 30 normal individuals, platelets were the only cell type stained for GPs IIb and IIIa. Monocytes were clearly not reactive with monospecific rabbit antisera against GP IIb or IIIa or two monoclonal antibodies specific for the platelet GP IIb/IIIa complex. Frequent significant fluorescence associated with mononuclear cells stained by immunofluorescence for GPs IIb and IIIa was attributable to platelets intimately associated with these cells. Figure 5 depicts mononuclear cell preparations stained for GP IIb/IIIa using monoclonal antibody preparation PC-1 (A and B) in which fluorescence staining is restricted to platelets (A′ and B′).

The expression of GPs IIb and IIIa by monocytes was studied further using complement-mediated cytotoxicity (Table 1). In this sensitive system, anti-Mol mediated the lysis of blood monocytes, but no monocyte cytolysis was observed with polyclonal and monoclonal antibodies to platelet GPs IIb, IIIa, or Ib or a heterologous polyspecific antiplatelet membrane preparation.

DISCUSSION

Studies described in this communication demonstrated that GPs IIb and IIIa associated with blood monocytes are derived from platelets. Previously, GP IIb/IIIa has been regarded as a restricted antigen for the megakaryocyte/platelet lineage.[8,18] However, the expression of GP IIb/IIIa by blood monocytes has been reported recently by several authors.[13,14,16] Preparations of blood monocytes with different degrees of platelet contamination were labeled in vitro with [^{35}S]methionine with the expectation that newly synthesized proteins would be monocyte-derived, since platelets contain only rudimentary protein synthetic systems.[33,34] Analyses of immunoisolated GPs IIb and IIIa from colabeled cultures of monocytes and platelets indicated that no GP IIb or IIIa was newly synthesized. On the other hand, substantial amounts of unlabeled GPs IIb and IIIa were detected and found to vary with the level of platelet contamination. The failure to detect synthesis of GP IIb or IIIa may be attributed to the lack of synthesis by mononuclear cells. Based on the 1 to 10 pg protein level of sensitivity described for fluorography,[27,28] in our system, monocytes could not have synthesized more than 0.076 to 0.76 GP IIb/IIIa molecules per monocyte. In addition, immunoprecipitated GP IIb/IIIa from mononuclear cells corresponded to about 9 to 18.5×10^{-6} of the total TCA precipitable material. Synthesis of GP IIb/IIIa, however, was demonstrated in human marrow megakaryocytes using the same experimental approach.[11] In 5×10^5 [^{35}S]methionine-labeled megakaryocytes, immunoprecipitated GP IIb/IIIa represented only 2×10^{-4} of all TCA precipitable counts, indicating that the detection system used was highly sensitive in detecting levels of approximately 0.01% to 0.02% of total precipitable counts.[11] Furthermore, while synthetic rates for specific proteins have not been determined in the mononuclear cell preparations studied, synthesis of an array of proteins inhibitable by cycloheximide was observed.

Studies conducted by several groups have demonstrated that platelets contain vestigial amounts of mRNA and may be capable of minimal protein synthesis.[35] In our studies using gel-filtered platelets and [^{35}S]methionine with high specific activity, we have detected trace amounts of protein synthesis comparable 1/10,000 of the specific incorporation observed in other blood cell types. However, attempts to immunoisolate metabolically labeled GPs IIb and IIIa from postculture preparations of gel-filtered or washed platelets failed to demonstrate neosynthesis of these antigens.

Immunofluorescence studies detecting GP IIb and IIIa molecules in blood monocyte preparations revealed that staining was restricted to platelets. Similarly, only platelets and megakaryocytes were stained in a series of 45 immunofluorescence analyses of peripheral blood and bone marrow

Table 1. Detection of Cell Membrane Antigens on Blood Monocytes by Complement-Mediated Cytolysis

Antibody Preparation	Mononuclear Cells Killed (%)
Polyspecific antiplatelet membrane	3
Heterologous antiplatelet GP IIb/IIIa	<1
Monoclonal antiplatelet GP IIb/IIIa (PC-1)	1
Heterologous antiplatelet GP Ib	<1
Monoclonal antiplatelet GP Ib	<1
Monoclonal anti-Mol	39
Normal rabbit IgG	<1
Normal mouse IgG	<1
Rabbit IgM antimouse IgG	3
Complement	2
Medium	1

Cytotoxicity was performed using a two-step assay (heterologous rabbit antiserum + complement) or a three-step assay (monoclonal antibody + rabbit IgM antimouse IgG + complement). Monocytes represented approximately 40% of the low-density mononuclear blood cells ($d < 1.077$ g/cm^3). Cell viability was assessed by trypan blue exclusion.

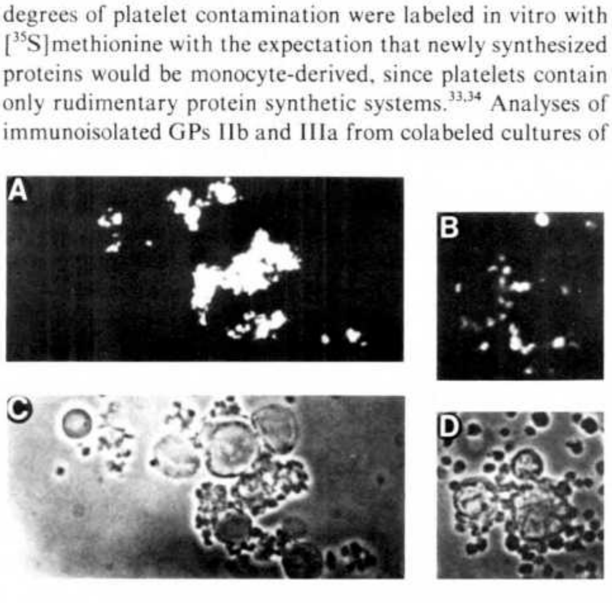

Fig 5. (A through D) Mononuclear blood cells stained by immunofluorescence after fixation with monoclonal antibody PC-1 specific for platelet GP IIb/IIIa and TRITC rabbit antimouse Fab Ig. Fluorescent panels (a and b) and phase-contrast counterparts (a′ and b′) demonstrate restriction of immunofluorescence staining to platelets (×595).

cell preparations using heterologous antisera monospecific for GPs IIb and IIIa as well as two monoclonal antibodies specific for the platelet GP IIb/IIIa complex. Furthermore, GPs IIb and IIIa present in mononuclear cell preparations failed to trigger complement-mediated lysis of monocytes in assays using heterologous and monoclonal antibodies specific for these antigens, indicating that these molecules were not integrally associated with monocyte plasma membranes.

Previously, Gogstad et al[13] have reported the detection of an antigen immunologically indistinguishable from the platelet GP IIb/IIIa complex in blood monocytes. In crossed immunoelectrophoretic analyses of preparations of solubilized monocyte membranes using a monospecific antiserum to the GP IIb/IIIa complex, Gogstad and associates demonstrated a single immunoprecipitate representing this molecular entity. However, adherent monocytes isolated by conventional techniques after full depletion of free-floating platelets typically have at least three to five platelets specifically bound to their surfaces and appear to represent the source of platelet GPs IIb and IIIa detected by Gogstad et al.[13] Moreover, their observation that no monocyte-associated GP IIb/IIIa was detectable in cells in a patient homozygous for Glanzmann's thrombasthenia does not clarify its source. Detection of the platelet GP IIb/IIIa complex in mononuclear cell preparations using a monoclonal antibody has been reported by Burckhardt et al.[14] However, high levels of platelet contamination are inferred from their experimental protocol. Removal of platelets adhered to monocytes requires extensive, repeated incubations with solutions containing an excess of 5 mmol/L EDTA and intervening differential centrifugations.[20] Isolation of platelet GPs IIb and IIIa from blood monocytes also has been reported by Bai et al[16] using two monoclonal antibody preparations against the GP IIb/IIIa complex. However, the mononuclear cell preparations were obtained from defibrinated peripheral blood, which is known to contain high concentrations of platelet microparticles.[36] Serum obtained from defibrinated blood contains platelet membrane fragments generated during cell activation with quantifiable levels of platelet proteins, including platelet GPs Ib, IIb, and IIIa, as well as platelet factor 4.[36] The description by Bai et al that GP Ib was readily recognized in their monocyte preparations is puzzling. Using the same monoclonal antibody (AN-51), other laboratories have not detected monocyte-associated GP Ib, suggesting that its presence may indeed represent contamination by platelet-derived proteins.[37] Expression of platelet GP IIb/IIIa by an erytholeukemia-derived cell line, K-562, expressing multilineage phenotypes, has been reported by Gewirtz et al[15] and Tabilio et al.[17] However, using the same and different antibody preparations, other authors failed to detect GPs IIb and IIIa in K-562 cells, indicating that gene expression of these antigens in neoplastic hematopoietic cell lines may be erratic.[38] HEL cell line established from a patient with Hodgkin's disease who later developed an erythroleukemia also expressed GPs IIb and IIIa.[39] Two other cell lines—U-937, derived from histiocytic lymphoma cells, and HL-60, from promyelocytes—have been reported to express GP IIb/IIIa.[14,16]

A number of similar and shared structural properties have been recognized between platelets and endothelial cells, including the expression of factor VIII:von Willebrand factor, fibronectin, thrombin receptors, and thrombospondin.[40-43] More recently, a GP IIIa-like molecule has been characterized in umbilical vein endothelial cells using a monoclonal antibody preparation.[44] Intriguingly, no GP IIb was co-isolated with this GP IIIa-like molecule, suggesting that certain differences exist between this new entity and the classical GP IIb/IIIa complex, as identified on platelets and megakaryocytes.[6] Structural homology between platelet GP IIIa and its endothelial analog may reflect common physiological functions or may be due to evolution from a common archetype.[45]

Characterization of GPs IIb and IIIa in normal hemopoietic cells by immunofluorescence have revealed that platelets and megakaryocytes are the only blood and marrow cell types expressing the GP IIb/IIIa complex.[7,8,10] The clonable progenitor for megakaryocytes (CFU-Mk)—but not the progenitors for the multilineage colonies (CFU-GEMM), erythroid (BFU-E), or the myelomonocytic (CFU-GM) lineages—contain the GP IIb/IIIa complex.[12] Additionally, synthesis of GPs IIb and IIIa with similar molecular characteristics as their platelet counterparts has been demonstrated in isolated human marrow megakaryocytes, thereby establishing the source of these glycoproteins in platelets.[11]

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Sulfhydryl Reducing Agents and Shape Regulation in Human Erythrocytes

By Hoai-Thu N. Truong, James E. Ferrell, Jr, and Wray H. Huestis

Metabolic crenation of red cells is reversible; on addition of nutrients, echinocytes recover the normal discoid shape. When the shape recovery takes place in the presence of reducing agents such as dithiothreitol (DTT), morphological change continues until the cells are stomatocytic. The degree of stomatocytosis varies, depending on the cell morphology when the nutrients and reducing agent are added. DTT has minimal effect on the shape of normal discocytes, but in its presence, mildly echinocytic cells become slightly cupped and advanced-stage echinocytes become severely stomatocytic. DTT must be present continuously for development and retention of stomatocytosis; echinocytes preincubated with or metabolically depleted in DTT do not become stomatocytic when supplemented in the absence of DTT, and DTT-induced stomatocytes revert to discocytes when the reducing agent is removed. DTT has no effect on adenosine triphosphate synthesis or equilibrium cell glutathione levels, and the induced stomatocytosis is not inhibited by excluding oxygen from cells during depletion. Spectrin phosphorylation and phosphate turnover are not affected by DTT. The echinocyte-to-discocyte transformation coincides with phosphorylation of membrane inner monolayer lipids (diacylglycerol to phosphatidic acid and phosphatidylinositol to phosphatidylinositol-4,5-bisphosphate). Overphosphorylation of these phospholipids is not responsible for the exaggerated shape recovery seen with reducing agents; phosphorylation of inner monolayer lipids proceeds identically in the presence and absence of DTT.

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THE NORMAL DISCOID SHAPE of the human erythrocyte can be altered in vitro by metabolic depletion,[1] calcium loading,[2] or addition of a variety of amphipathic agents.[3] Control of normal cell shape has been attributed to the protein cytoskeleton that laminates the cytoplasmic face of the membrane,[4,5] with the suggestion that phosphorylation and dephosphorylation of spectrin could be the mechanism by which the cell regulates its morphology.[6,7] While an intact cytoskeleton is necessary for shape changes to occur,[8] recent work suggests that its role is passive[9,10] in that it adopts the conformation imposed by the bilayer.

Another model for control of red cell shape is the bilayer couple hypothesis,[11] which attributes cell shape changes to alterations in the relative areas of the inner and outer monolayers of the cell membrane. Any event or external agent that causes the outer monolayer to expand relative to the inner monolayer produces spiked spheres, echinocytes. Likewise, preferential expansion of the inner leaflet results in formation of cupped cells and spheres with invaginations (stomatocytes), species that appear to be the reverse of echinocytes. Agents that induce stomatocytosis can reverse the effects of echinocytic agents and vice versa.

Metabolic crenation is thought to result from bilayer imbalance secondary to dephosphorylation of phosphatidylinositol-4,5-bisphosphate and phosphatidic acid.[12] This crenation eventually becomes irreversible, but for many hours after adenosine triphosphate (ATP) depletion, addition of nutrients will induce echinocytes to revert to discocytes. The shape recovery coincides with phosphorylation of inner-membrane phospholipids that were dephosphorylated during crenation.[12,13]

Palek et al[14] report that the reducing agent dithiothreitol accelerates the shape recovery of metabolically crenated cells, perhaps by reducing cross-linked cytoskeletal elements. In this paper, we report that DTT and other reducing agents effect further shape changes in intact cells, inducing them to form stomatocytes rather than reaching a stable discoid shape. The biochemical consequences of such reduction are examined.

MATERIALS AND METHODS

Blood was obtained from healthy adult volunteers by venipuncture and collected in EDTA, heparin, or citrate anticoagulant. Erythrocytes were separated from plasma and buffy coat by centrifugation at 1,800 g for five minutes and washed three times in 4 vol of 150 mmol/L NaCl. Cells were used within six hours of being drawn. If the erythrocytes were not used immediately after washing, they were suspended in 138 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L NaH_2PO_4, 6.1 mmol/L Na_2HPO_4, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, pH 7.4, and stored at 4 °C. Such cells were washed once in 150 mmol/L NaCl immediately before the experiment.

$[^{32}P]H_3PO_4$ (carrier-free) was purchased from ICN Radiochemicals (Irvine, Calif). Penicillin G was from Pfizer (New York). Luciferin, luciferase, 3,3'-dithiobis(6-nitrobenzoic acid) (DTNB), and DTT were obtained from Calbiochem-Behring (La Jolla, Calif). DTT was also purchased from Sigma (St Louis), which was the source of all other biochemicals. Other chemicals, obtained from J.T. Baker Chemical Co (Phillipsburg, NJ) or Fischer Scientific Co (Pittsburgh), were of at least reagent grade.

Film for autoradiography was obtained from Eastman Kodak Co (Rochester, NY); Cronex Lightning Plus intensifying screens were from DuPont Instruments (Wilmington, Del). Silica Gel HL plates were purchased from Analtech Inc (Newark, Del).

Metabolic Depletion and Reversal

All incubations were carried out at 37 °C in capped plastic tubes. The incubation buffers contained 100 μg/mL penicillin and 100 μg/mL streptomycin to retard bacterial growth. To induce ATP depletion and crenation, cells were incubated in 4 vol of 150 mmol/L NaCl, 7.5 mmol/L Na_2HPO_4, pH 7.4, at 22 °C ($NaCl/P_i$). Anaerobic depletion was carried out in sealed tubes containing humidified N_2. The inert gas was bubbled through the cell suspension every ten hours or when tubes were opened for sampling, whichever was more frequent.

Shape recovery was effected by resuspending the pelleted echinocytes in 4 vol of 138 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L NaH_2PO_4, 6.1 mmol/L Na_2HPO_4, 1 mmol/L $MgSO_4$, 5 mmol/L

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glucose, pH 7.4, supplemented with an additional 5 mmol/L glucose, 10 mmol/L inosine, and 1 mmol/L adenosine (supplemented NaCl/P_i). Stomatocyte formation was induced by adding DTT at a concentration of 10 mmol/L unless otherwise noted.

In some experiments, crenated cells were diluted to a hematocrit of 10% with NaCl/P_i and *N*-tosyl-L-phenylalanine chloromethyl ketone (TosPheCH$_2$Cl, 200 mmol/L in dimethylsulfoxide) was added to yield a final concentration of 0.4 mmol/L. Control cells were treated similarly with dimethylsulfoxide alone.

In spectrin phosphorylation experiments, cells were prelabeled in 1 vol of 10 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1 mmol/L MgSO$_4$, 1 mmol/L K$_2$HPO$_4$, 5 mmol/L KCl, 137 mmol/L NaCl plus 50 μCi/mL [^{32}P]H$_3$PO$_4$ (HEPES/$^{32}P_i$), supplemented with 10 mmol/L inosine, 10 mmol/L glucose, and 1 mmol/L adenosine. After ten hours the suspension was replenished with 10 mmol/L inosine, 10 mmol/L glucose, and 1 mmol/L adenosine. After 23 hours' incubation, by which time the amount of ^{32}P in spectrin reaches a steady state,[15] cells were pelleted, resuspended in HEPES/$^{32}P_i$ of the same specific activity, and allowed to deplete metabolically. Reversal of metabolic depletion was carried out in the same HEPES/$^{32}P_i$, supplemented with sugars. Turnover and rephosphorylation of spectrin were examined in cells depleted in ^{32}P-free NaCl/P_i and resuspended in supplemented HEPES/$^{32}P_i$.

In lipid phosphorylation experiments, cells were labeled with ^{32}P by depleting them in 1 vol of NaCl/P_i plus 150 μCi/mL [^{32}P]H$_3$PO$_4$. After approximately 24 hours of incubation at 37 °C, the cell suspension was diluted into 10 mmol/L Tris, 150 mmol/L NaCl (pH 7.4 at 22 °C), 10 mmol/L glucose, 10 mmol/L inosine, and 1 mmol/L adenosine to give a final hematocrit of 20%.

Assay Procedures

Morphology. Erythrocytes were diluted to a hematocrit of 5% and fixed in 150 mmol/L NaCl containing 0.5% glutaraldehyde. Cells were examined by bright field microscopy at a magnification of ×500. Stomatocytes were assigned a score of -4 to -1,[16] discocytes a score of 0, and echinocytes a score of $+1$ to $+5$.[12] The average score for a field of 100 cells was called its morphological index (MI).[17] Some fixed cell samples were washed with distilled water, placed on glass coverslips, air-dried, and examined by scanning electron microscopy.

Adenosine triphosphate. Frozen aliquots of cell suspension (50 μL) were diluted in 2.45 mL 150 mmol/L NaCl, boiled for 15 minutes, and centrifuged to clarity. The supernatant (undiluted or diluted 1:10) was assayed for ATP by the luciferin-luciferase method.[18]

Reduced glutathione. Reduced glutathione was measured by the DTNB reagent method[19] on frozen acid lysate of cells, unless otherwise noted. When present, DTT was removed from cell suspensions by repeated washing to remove the DTT, which also reacts with DTNB.

Spectrin phosphorylation. Aliquots (150 μL) of 20% cell suspension were lysed in 1.2 mL 10 mmol/L Tris, 2 mmol/L EDTA, pH 7.5, at 22 °C immediately after removal from incubation. The stroma were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5% slab gels.[20] Autoradiograms of the dried gels were obtained by one-day exposures to Kodak X-OMAT AR film with intensifying screens. Individual bands of the gels were cut out, dissolved in 1 mL 30% H$_2$O$_2$ at 60 °C for nine to 24 hours, and analyzed by liquid scintillation counting. Results were normalized to the amount of protein loaded onto the gel, as determined by the Lowry assay.[21]

Lipids. Aliquots (100 μL) of cell suspension were removed during the shape recovery and stored frozen. Phosphatidylinositol-4-phosphate, phosphatidylinositol-4,5-bisphosphate, and phosphatidic acid levels were measured as described previously.[12] The frozen aliquots were lysed in 2 mL cold 10 mmol/L Tris, 2 mmol/L EDTA (pH 7.5 at 22 °C) and washed once in the same buffer. The pellet was extracted with 1.8 mL methanol/chloroform/concentrated HCl, 100:50:1 (vol:vol:vol), followed by water (0.6 mL) and chloroform (0.6 mL). Aliquots (0.7 mL) from the organic phase were dried under a stream of N$_2$ and redissolved in three 5-μL portions of chloroform. Lipids were separated by thin-layer chromatography on nonactivated 2.5 × 10-cm plates in 48:40:10:5 (vol:vol) methanol/chloroform/H$_2$O/ concentrated NH$_3$. Lipid spots were detected by iodine vapor and autoradiography. ^{32}P-labeled phospholipids were quantified by liquid scintillation counting of scraped spots.

RESULTS

Erythrocytes were incubated in NaCl/P_i to effect metabolic depletion. As shown previously,[1,12] ATP depletion proceeded with a half time of five to ten hours and was followed by metabolic crenation. Metabolically depleted echinocytes (Fig 1A) were pelleted and resuspended in sugar supplemented NaCl/P_i. ATP levels were partially restored and the cells recovered normal discoid shape, remaining in this configuration for many hours as long as nutrients were available (Fig 1B and Fig 2). DTT altered this shape recovery dramatically. In the presence of 10 mmol/L DTT, echinocytes quickly recovered discoid shape and then became cupped and invaginated stomatocytes (Fig 1C and Fig 2). The extent of the morphological overshoot depended on the original extent of crenation. Stage 1 echinocytes attained a slightly cupped shape, while severely crenated cells eventually developed the membrane invaginations of advanced-stage stomatocytes (Fig 3A). Similarly, if echinocytes had partially recovered toward discoid shape before addition of the reducing agent, the extent of DTT-induced stomatocytosis diminished (Fig 3B).

Other sulfhydryl reducing agents also induced stomatocytosis in recovering echinocytes but to variable extents. Dithioerythritol produced effects similar to the DTT response and in the same concentration range. 2-Mercaptoethanol induced detectable but less severe stomatocytosis; even at concentrations greater than 50 mmol/L this reagent typically produced morphological indices no greater than -1.0 (data not shown). Echinocytes treated with sodium dithionite (Na$_2$S$_2$O$_4$, 10 mmol/L) did not recover discoid or stomatocytic morphologies in the presence of sugars; thus, some reducing agents inhibit even normal shape recovery.

The continuous presence of the reducing agent was required to generate and maintain stomatocytic morphologies. Echinocytes pretreated with DTT (10 mmol/L for two hours at 0 °C) and then washed free of DTT before nutrient supplementation recovered the normal discoid shape but did not cup (Fig 4A). Identical results were obtained with DTT pretreatment at 37 °C. When DTT-induced stomatocytes were washed free of the reducing agent, the cells slowly recovered discoid shape (Fig 4B).

The extent of induced stomatocytosis was dependent on the DTT concentration. At hematocrits of 5% and 20%, half-maximal stomatocytosis was obtained at DTT concentrations of 2 to 3 mmol/L (Fig 5A). The same concentration dependence was observed for cells beginning at different

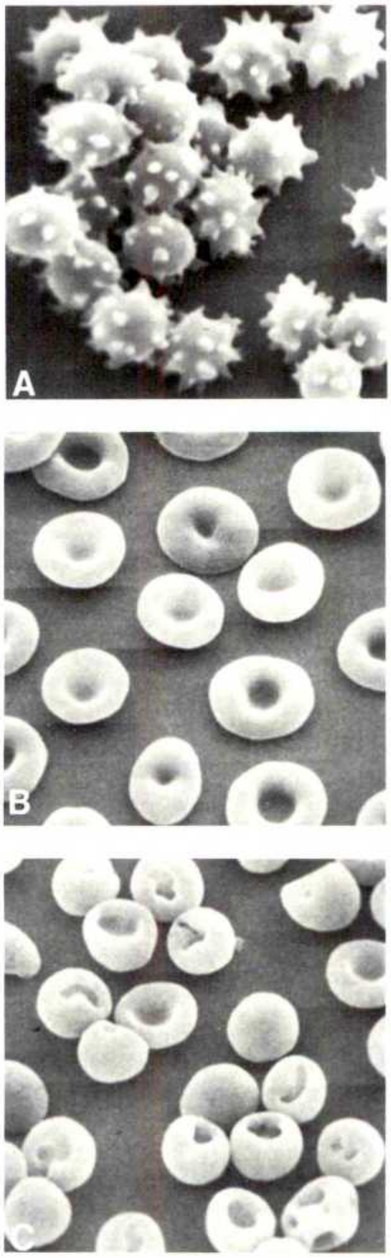

Fig 1. Scanning electron micrographs of (A) echinocytes formed by 21 hours of metabolic depletion; (B) discocytes obtained by incubating depleted cells for four hours in supplemented NaCl/P$_i$; (C) stomatocytes resulting from incubating depleted cells in supplemented NaCl/P$_i$ containing 10 mmol/L DTT. Original magnification ×3,000.

stages of crenation. At three points during metabolic depletion, cells were pelleted and resuspended in supplemented NaCl/P$_i$ with DTT at concentrations ranging from 0 mmol/L to 10 mmol/L. Half-maximal overshoot occurred at 2 to 3 mmol/L DTT with samples whose initial morphological indices were +0.9, +1.6, or +2.1 before supplementation (Fig 5B).

Oxidative damage. To examine the role of oxidative damage in DTT-induced stomatocytosis, cells were depleted under an inert atmosphere and in air. The inert atmosphere slowed glutathione depletion (Fig 6) but did not alter the rate of crenation. On sugar supplementation, erythrocytes depleted under an inert atmosphere recovered the normal discoid shape more rapidly than cells depleted under air, as reported previously.[14] When supplemented in the presence of DTT, cells depleted under both conditions formed stomatocytes at the same rate and to the same extent (Fig 7).

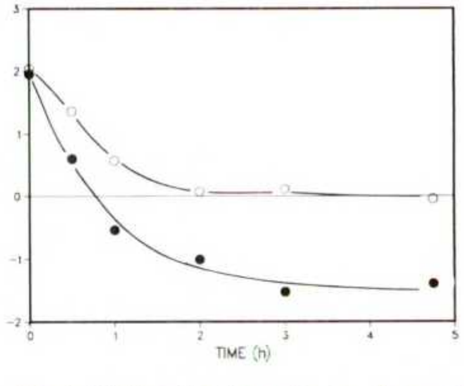

Fig 2. Effect of DTT on the shape recovery of crenated cells. Erythrocytes were metabolically depleted for 24 hours and then resuspended in supplemented NaCl/P$_i$ (○) or in supplemented NaCl/P$_i$ plus 10 mmol/L DTT (●).

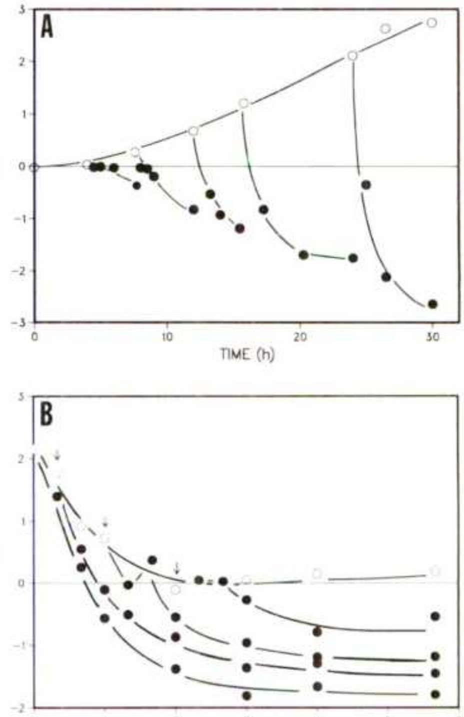

Fig 3. DTT-induced stomatocytosis at various stages of crenation. (A) Cells were metabolically depleted (○) in NaCl/P$_i$ + 10 mmol/L MgSO$_4$. At t = 4, 8, 12, 15.75, and 22 hours, aliquots were removed and incubated with supplemented buffer containing 10 mmol/L DTT (●). (B) Cells were metabolically depleted for 22 hours, pelleted, and then resuspended in supplemented NaCl/P$_i$ (○). At t = 0, 0.33, 0.67, 1, and 2 hours after resuspension, aliquots were removed and incubated with supplemented NaCl/P$_i$ containing 10 mmol/L DTT (●).

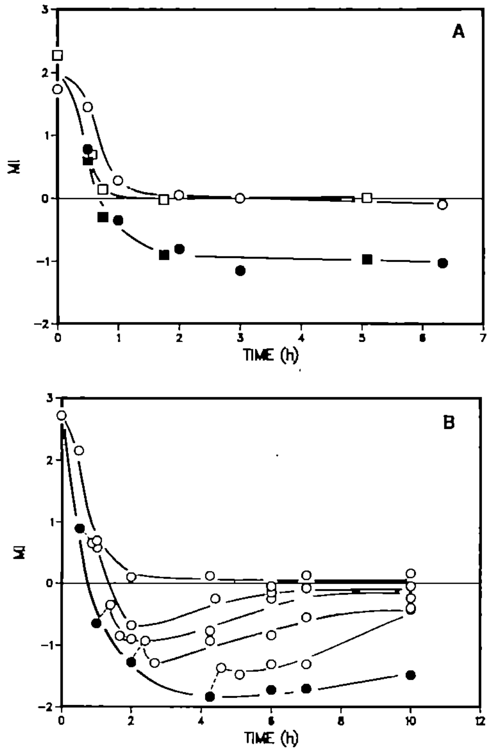

Fig 4. The continuous presence of DTT is required for stomatocytosis. (A) Cells were metabolically depleted for 24 hours. Cells were treated with 10 mmol/L DTT at 0 °C for two hours and washed repeatedly (□, ■) or not treated (O, ●). Cells were then resuspended in supplemented NaCl/P_i, without DTT (O, □) or with 10 mmol/L DTT (●, ■). (B) Erythrocytes were depleted for 23 hours and resuspended in supplemented NaCl/P_i without DTT (O) or with 10 mmol/L DTT (●). At t = 0.5, 1, 2, and 4.25 hours after resuspension in the DTT-containing buffer, aliquots were removed, washed extensively, and reincubated in DTT-free supplemented NaCl/P_i (O).

Similarly, cells depleted in 2 mmol/L DTT and then washed to remove the reducing agent recovered discoid shape. In the presence of additional DTT, they formed stomatocytes at the same rate and to the same extent as control cells (data not shown).

N-tosyl-L-phenylalanine chloromethyl ketone experiments. The chloromethyl ketone, TosPheCH$_2$Cl accelerates the shape recovery of echinocytes obtained by incubation with dinitrophenol.[22] TosPheCH$_2$Cl did not alter the shape recovery of metabolically crenated cells, but in its presence, DTT induced greater than normal stomatocytosis (Fig 8).

ATP and glutathione. Glutathione and ATP levels were assayed during shape recovery. ATP levels typically recovered to about 40% of their normal value, both in control and DTT-treated cells (Fig 9). Glutathione levels recovered to approximately 50% of the value found in fresh cells. DTT reduced all available oxidized glutathione within 20 minutes (the minimum time to prepare samples for the glutathione assay), but it had no effect on the final concentrations of reduced glutathione (Fig 9).

Spectrin phosphorylation. Spectrin, the major cytoskeletal protein, is phosphorylated in the band 2 subunit. The level of phosphorylation decreases after metabolic depletion and crenation and increases again when sugars are added.[15] Spectrin phosphorylation was not altered in echinocytes recovering in the presence of DTT. Cells prelabeled with ^{32}P were metabolically depleted and then resuspended in sugar

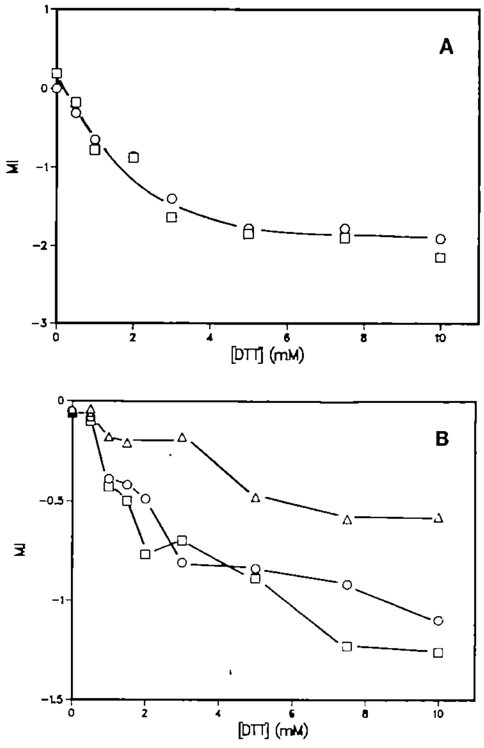

Fig 5. Concentration dependence of DTT stomatocytosis. (A) Effect of hematocrit. Cells were metabolically depleted for 21 hours, pelleted, and resuspended at a hematocrit of 5% (□) or 20% (O) in supplemented NaCl/P_i with [DTT] ranging from 0 to 10 mmol/L, and incubated for 4.5 hours. (B) Effect of degree of crenation. Cells were metabolically depleted. After 14 hours (MI = +0.9) (Δ), 21 hours (MI = +1.6) (O), and 24 hours (MI = +2.1) (□) of depletion, aliquots of crenated cells were removed, pelleted, resuspended at a hematocrit of 20% in supplemented NaCl/P_i with [DTT] ranging from 0 to 10 mmol/L, and reincubated for four hours.

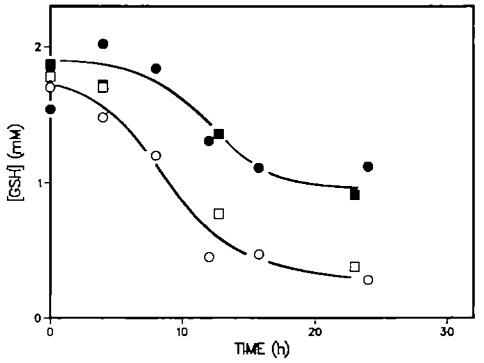

Fig 6. Glutathione levels during metabolic crenation under an inert atmosphere. Cells were depleted in air (O, □) or under humidified N_2 (●, ■). Data from two similar experiments are shown. Glutathione levels were measured on frozen cell suspensions.

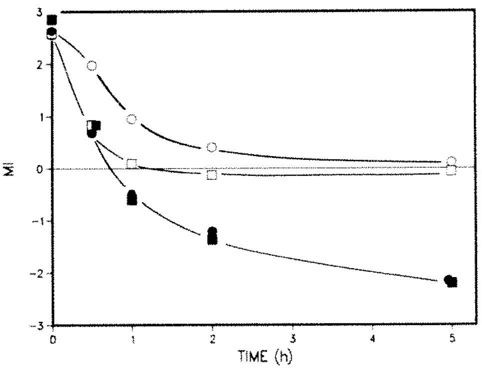

Fig 7. **Effect of prevention of oxidative damage during depletion. Cells were metabolically depleted in air (○, ●) or under N_2 (□, ■) for 23 hours and then incubated with sugar-supplemented medium with (●, ■) or without (○, □) 10 mmol/L DTT.**

supplemented buffer containing $^{32}P_i$ at the same specific activity as the buffer used for labeling and depleting the cells. DTT did not affect the rate or extent of spectrin phosphorylation (Fig 10A); thus, spectrin phosphate levels were not altered by the reducing agent. To determine whether spectrin phosphate turnover is linked to DTT-induced stomatocytosis, cells were metabolically depleted in ^{32}P-free buffer. The resulting echinocytes were resuspended in sugar-supplemented buffer containing ^{32}P. The ensuing incorporation of ^{32}P into spectrin should be due both to spectrin rephosphorylation and to turnover of residual phosphate groups. DTT had no effect on phosphate incorporation under these conditions (Fig 10B); thus, it does not alter the rate of turnover of spectrin phosphate.

Phosphoinositide and diacylglycerol phosphorylation. Metabolic crenation of erythrocytes coincides with the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol and of phosphatidic acid to diacylglycerol. Lipid dephosphorylation is reversed by the addition of sugars and accompanies the shape recovery.[12] The phosphorylation of these lipids was examined in DTT-induced stomatocytes. Cells were metabolically depleted in the presence of $^{32}P_i$. The resulting echinocytes were incubated with sugars in the presence and absence of DTT. The shape recovery to discocyte and the DTT-induced stomatocyte formation were both accompanied by an increase in the ^{32}P incorporated into phosphatidylinositol-4,5-bisphosphate, a decrease in phosphatidylinositol-4-phosphate levels and no change in the levels of phosphatidic acid (Fig 11). DTT had no effect on the amount of ^{32}P incorporated into phosphatidylinositol-4,5-bisphosphate, phosphatidylinositol-4-phosphate, phosphatidic acid, or any other phospholipid.

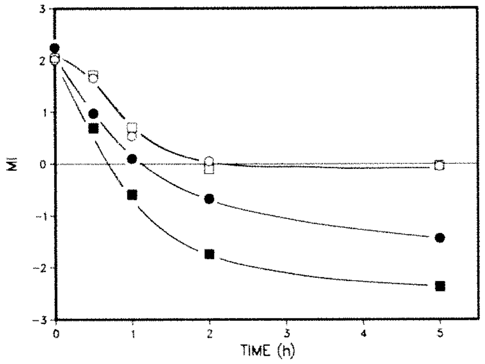

Fig 8. **Effect of TosPheCH$_2$Cl on DTT-induced stomatocytosis. Cells were metabolically crenated for 22 hours, treated with dimethyl sulfoxide (○, ●) or with TosPheCH$_2$Cl in dimethyl sulfoxide (□, ■), and then resuspended in supplemented NaCl/P_i without (○, □) or with 10 mmol/L DTT (●, ■).**

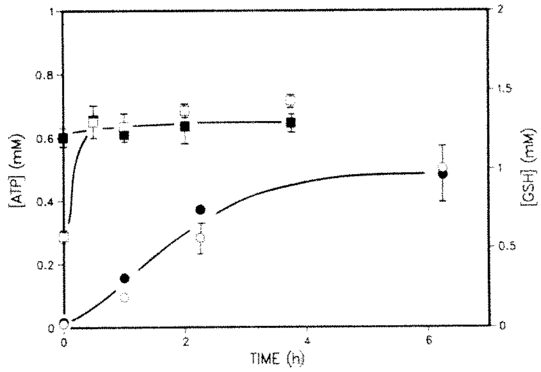

Fig 9. **ATP and glutathione levels during shape recovery. Data from two experiments are shown. Cells were metabolically depleted for 23 hours for GSH measurements (□, ■), or for 25 hours for ATP measurements (○, ●). Recovery was carried out in NaCl/P_i without (○, □) or with 10 mmol/L DTT (●, ■). Values are the average of two measurements. Error bars in this and all subsequent figures represent one standard error.**

DISCUSSION

The sulfhydryl reducing agent DTT has no evident effect on the morphology of normal human discocytes or echinocytes generated by metabolic depletion. However, when DTT is present during ATP repletion, echinocytes recover, not to discocytes, but to stomatocytes. The severity of the stomatocytosis is a function of the degree of initial crenation; more severely echinocytic cells cup more extensively. This is true both for cells depleted for varying lengths of time and for partially recovered echinocytes incubated in supplemented medium for varying lengths of time. Thus, DTT intervenes in some way in an ATP-dependent mechanism that restores and regulates cell shape.

Oxidative damage of cell components. The stability of ghosts and of cytoskeletal protein complexes depends on the integrity of sulfhydryl groups in actin and band 3.[23] Palek et al[14] found that DTT accelerates sugar-dependent shape recovery of metabolically depleted echinocytes.* They proposed that the cytoskeleton undergoes oxidative damage during depletion and that such damage must be repaired during repletion before the cell can assume its normal shape. These considerations raised the possibility that DTT affects cell shape by reducing certain protein sulfhydryl groups

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*These authors do not report finding stomatocytes in the population of recovered cells. The discrepancy between their results and the present findings may be due to differences in experimental conditions or in the method of assigning cell morphology.

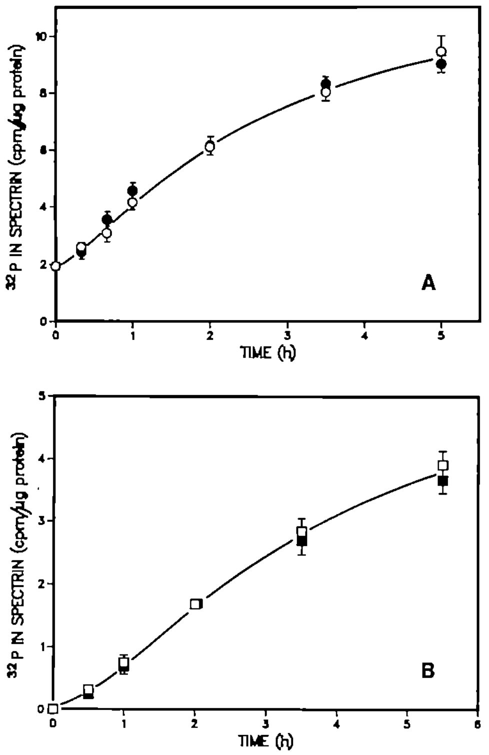

Fig 10. Spectrin phosphorylation and DTT-induced stomatocytosis. (A) Net phosphorylation in the band 2 region. Cells were pre-labeled with ^{32}P, metabolically depleted for 32 hours, pelleted and resuspended in sugar-supplemented ^{32}P-containing buffer with (●) or without 10 mmol/L DTT (○); $t_{1/2}$ of shape recovery – 1.5 hours. Values are the average of three measurements. (B) Rephosphorylation and turnover. Cells were depleted for 23 hours, pelleted, and resuspended in sugar-supplemented ^{32}P-containing buffer with (■) or without 10 mmol/L DTT (□); $t_{1/2}$ of shape recovery – two hours. Values are the average of four measurements.

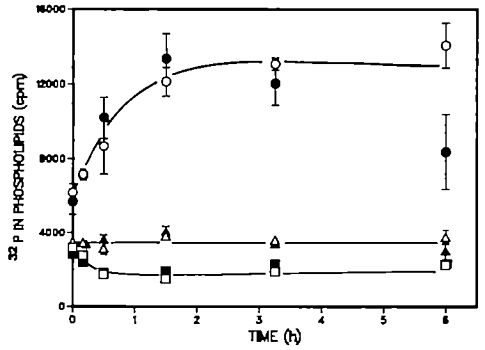

Fig 11. Phosphoinositide metabolism and DTT-induced stomatocytosis. Cells were metabolically depleted in ^{32}P-containing buffer for 21 hours and then diluted in sugar-supplemented buffer with or without 10 mmol/L DTT; $t_{1/2}$ of shape recovery – two hours. Phosphatidylinositol bisphosphate (○, ●), phosphatidylinositol monophosphate (□, ■), and phosphatidic acid (△, ▲). No differences in any lipid concentration were observed in the absence (○, □, △) or presence (●, ■, ▲) of 10 mmol/L DTT. Values are the average of four measurements.

beyond the extent normally attained in metabolic processes, releasing oxidative constraints to an abnormal degree. If the susceptibility of these proteins to such alteration were increased by the putative oxidative damage, the DTT effect might manifest itself, as observed, only in cells recovering from crenation.

This possibility was examined in experiments in which cells were allowed to crenate in the absence of oxygen or in the continuous presence of DTT. Neither of these treatments affects the degree of stomatocytosis observed when the echinocytes were supplemented with sugars and DTT. Alternatively, echinocytes generated under the usual experimental conditions were incubated for two hours with DTT and then supplemented with nutrients and allowed to change shape in the absence of DTT. These cells recover discocytic shape but do not become stomatocytic. Thus, protection of cells from oxidative damage does not abolish the effects of DTT.

Time course and concentration dependence of DTT responses. DTT must be present continuously not only for stomatocyte formation, but also for maintenance of the stomatocytic shape. When the reducing agent is removed, stomatocytes slowly revert to discocytes. This "relaxation" occurs on a longer time scale than the initial stomatocyte formation, indicating that the cell is slow to overcome the effects of DTT. The relaxation observed might be a reversal of the stomatocytic process, occurring at a slower rate because the cell's metabolic activity has reached a steady state; or it might involve a separate mechanism. It is perhaps significant that the time scale of stomatocyte reversal approaches the time scale of phospholipid flip-flop.[24]

DTT does not act in a stoichiometric fashion. Half-maximal stomatocytosis is observed at 2 to 3 mmol/L DTT, regardless of the initial degree of crenation or the cell concentration. This finding, together with the requirement for continuous presence of DTT, is more consistent with modulation of an enzyme activity than with direct reductive action on cytoskeletal protein structure.

Other reducing agents. Sulfhydryl reducing agents other than DTT induce stomatocyte formation in metabolically depleted echinocytes. Dithioerythritol effects stomatocytosis at the same concentration and to the same extent as does DTT. 2-Mercaptoethanol also induces stomatocyte formation but to a lesser extent, even at tenfold higher concentrations. The relative effects of mercaptoethanol and the bivalent reagents may reflect differences in their reduction potentials. The anomalous electrode behavior of thiols in aqueous solutions has made reliable determination of their relevant electrode potentials difficult, but relative values for mercaptoethanol and DTT may be inferred from their equilibrium constants for reduction of protein sulfhydryl groups: the value for mercaptoethanol is approximately unity, 10^4 less than that for DTT and dithioerythritol.[25]

Metabolic effects. ATP and glutathione are metabolic intermediates depleted during crenation and regenerated as cells return to discocytes. Neither process appears to be the target of DTT. The rate and extent of ATP resynthesis are not affected by DTT. Cells depleted under nitrogen retain higher levels of reduced glutathione than cells depleted in air, but DTT induces stomatocytosis in cells of both types at the same rate and to the same extent. DTT reduces oxidized glutathione within the first 20 minutes of incubation, but normal metabolic processes restore control cells to the same level well before stomatocytosis develops, and DTT does not alter the final level of reduced glutathione in the cell.

Protein phosphorylation. The rephosphorylation of spectrin, once thought to be the event effecting the echinocyte-to-discocyte transformation, is not altered during the echinocyte-to-stomatocyte transformation. DTT does not change the rate of spectrin rephosphorylation, nor does it alter phosphate turnover in band 2. No differences in phosphorylation of other membrane proteins are observed in DTT-treated and control cells.

Phosphoinositide metabolism and bilayer balance. The discocyte-to-echinocyte transformation in red cells is coupled to the metabolism of cell phosphoinositides.[12] During metabolic depletion, crenation coincides with the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol and phosphatidic acid to diacylglycerol. The reversal of the shape change by the addition of nutrients reverses this lipid dephosphorylation. Phosphoinositide phosphorylation is not responsible for DTT stomatocytosis; the rate of formation and equilibrium levels of phosphatidylinositol-4,5-bisphosphate and phosphatidic acid are indistinguishable in recovered discocytes and in DTT-induced stomatocytes. If the apparent connection between phosphoinositide phosphorylation and cell shape is due simply to steric effects on membrane bilayer balance, as suggested earlier,[12,13] the present findings would indicate that more than one metabolic mechanism influences cell shape. The morphology change from echinocyte to discocyte to stomatocyte has been viewed as a continuum, as in the case of amphipath intercalation. If metabolically mandated shape changes are also governed by a single biochemical mechanism, it is more complex than simple phosphoinositide occupancy of inner monolayer area.

From presently available evidence, DTT-induced stomatocytosis could be a bilayer effect, a cytoskeletal effect, or both. The time scale of the process is consistent with the rate of phosphatidylserine translocation across the bilayer.[16,26] Possibly, DTT activates a phospholipid translocation protein, causing net transfer of phospholipid to the inner monolayer. Alternatively, DTT may directly or indirectly alter cytoskeletal protein interactions, either among themselves or with the bilayer by way of the formation of a metabolite such as a polyanion.[7,27-29] Our preliminary results may support the last hypothesis. Conditions that elevate cell 2,3-diphosphoglycerate levels during shape recovery induce additional morphological anomalies, which are under further investigation.

Cells incubated with the amphipath dinitrophenol crenate, presumably due to the intercalation of dinitrophenol into the cell outer monolayer.[30] The echinocytes generated by this agent revert slowly to discocytes in the presence of glucose. This shape recovery is accelerated by TosPheCH$_2$Cl and hexose monophosphate shunt activators in normal cells,[22,31] an effect not found in glucose-6-phosphate-dehydrogenase–deficient cells. The shape recovery from dinitrophenol crenation may be related to DTT-induced stomatocytosis. TosPheCH$_2$Cl has no effect on the shape reversion of echinocytes to discocytes, but it potentiates the stomatocytic effect of DTT. This provides additional evidence that enzymes of the metabolic cycles are targets for the reducing agents.

Morphology control in ghosts. Under some conditions, ghosts resealed around physiological concentrations of MgATP form echinocytes that gradually become discoid and then stomatocytic.[6,7,32,33] These shape changes coincide with formation of phosphatidic acid,[34] and phosphatidylinositol-4,5-bisphosphate may also be elevated under some conditions.[35] The degree of stomatocytosis eventually attained in ghosts depends on the degree of washing they receive as well as the buffer and temperature used: greater stomatocytosis and endocytosis are observed with ghosts washed extensively before resealing (unpublished observations, 1984). It is possible that ghosts and DTT-treated echinocytes share the same biochemical lesion that prevents them from maintaining discoid shape.

ACKNOWLEDGMENT

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Localization of Factor VIII-Procoagulant Antigen: An Immunohistological Survey of the Human Body Using Monoclonal Antibodies

By Theo H. v.d. Kwast, Herbert V. Stel, Ellen Cristen, Rogier M. Bertina, and Enno C.I. Veerman

Various organs, including liver, spleen, heart, lung, kidney, intestines, lymph nodes, pancreas, bone marrow, and thymus, were investigated for the presence of factor VIII-procoagulant antigen (VIIICAg) and factor VIII-related antigen (VIIIRAg), using a panel of monoclonal antibodies directed to factor VIII-von Willebrand factor in combination with a sensitive immunoperoxidase staining technique. In addition to hepatic sinusoidal endothelial cells, the presence of VIIICAg was demonstrated in mononuclear cells sporadically present in lymph nodes, in the alveolar septa of lung, and in the red pulp of spleen. The identity of these mononuclear cells could not be unequivocally determined. Based on morphological criteria, however, it is tentatively concluded that these cells are nonlymphoid and belong to the mononuclear phagocyte system. The presence of VIIIRAg was confined to vascular endothelial cells, hepatic sinusoidal endothelial cells, cells lining the venous sinuses of the red pulp of the spleen, cells lining renal glomeruli and lung capillaries, platelets, and megakaryocytes.

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FACTOR VIII-von Willebrand factor (FVIII-vWF), a high-molecular-weight glycoprotein complex that has an important role in hemostasis, consists of two discernible components. These are factor VIII, the procoagulant component absent in severe hemophilia A, and von Willebrand factor (vWF), the component that is absent in severe von Willebrand's disease.[1] Immunohistological[2] and recombinant DNA studies[3-5] have indicated that factor VIII is present in and synthesized by liver cells. From earlier transplantation and perfusion studies,[6-8] circumstantial evidence was obtained that the liver is involved in the synthesis of factor VIII; however, a variety of other organs have been implicated in the synthesis of factor VIII as well.[9-11] Hence the possibility of extrahepatic synthesis of factor VIII cannot be excluded in advance. This prompted us to extend the immunohistological search for VIIICAg to various other organs and cell types by use of a panel of monoclonal antibodies with different specificity to factor VIII. In addition, parallel experiments were performed using monoclonal antibodies against vWF.

It appeared that the presence of factor VIII was restricted to two cell types: hepatic sinusoidal endothelial cells and mononuclear cells, found sporadically but consistently in lung, spleen, and lymph nodes.

MATERIALS AND METHODS

Monoclonal antibodies. For this study eight monoclonal antibodies to factor VIII and four monoclonal antibodies to vWF were used. Description of techniques for characterization of these antibodies have been provided elsewhere and are applicable for this study.[2,12] Some of these antibodies have already been described in detail elsewhere.[2,13-16] In brief, monoclonal antibodies CLB-CAg A (IgG_{2a}), CLB-CAg 21 (IgG_{2a}), CLB-CAg 44 (IgG_{2a}), CLB-CAg 58 (IgG_{2a}), CLB-CAg 61 (IgG_1), CLB-CAg 78 (IgG_1), CLB-CAg 117 (IgG_{2a}), and C 6 (IgG_1, IgG_{2b}) are directed to factor VIII as judged by the following criteria: they bind to purified FVIII-vWF[17] and to FVIII-vWF present in normal plasma, but they do not react with severe hemophilia A plasma (negative for material cross-reacting with a human antibody to VIIICAg). Some of these antibodies inhibit factor VIII-procoagulant activity (VIIIC): C6 (5,000 Bethesda units (BU)/mg IgG), CLB-CAg A (150 BU/mg IgG), CLB-CAg 58 (5 BU/mg IgG), and CLB-CAg 117 (2 BU/mg IgG), whereas the other monoclonal antibodies do not inhibit VIIIC. All these monoclonal antibodies react with serum VIIICAg, exept antibody C6, which suggests that the C6 antibody is specific for the native conformation of the factor VIII molecule. As deduced from the results of competitive displacement studies carried out essentially as described by Stel et al,[15] these antibodies were directed to different epitopes on factor VIII. Monoclonal antibodies CLB-RAg 1 (IgG_1), CLB-RAg 7 (IgG_1), CLB-RAg 35 (IgG_{2a}), and CLB-RAg 50 (IgG_1) are directed to vWF[15,16]: they bind to purified FVIII-vWF, to FVIII-vWF present in normal plasma, and to FVIII-vWF present in severe hemophilia A plasma. In contrast, these antibodies do not react with plasmas of patients with severe von Willebrand's disease (negative for material cross-reacting with a rabbit antibody to vWF). Competitive displacement studies showed that these antibodies are directed to different epitopes on vWF.

Immunoperoxidase staining procedure. A sensitive multistep immunoperoxidase staining method was used to demonstrate the presence of VIIICAg. This procedure has been described in detail by Lansdorp et al.[18] Sheep antimouse immunoglobulin (SAM) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam) was used to link peroxidase-mouse monoclonal antiperoxidase complexes (PAP) (CLB) to the initial monoclonal antibody (eg, monoclonal antibody to VIIICAg). The multistep PAP procedure was based on repeated subsequent incubation cycles with SAM and PAP complexes, respectively. The experimental details of the immunostaining procedure were as follows: (1) cryostat sections of 5-μ thickness were air-dried, fixed in acetone for ten minutes, and washed in phosphate-buffered saline (PBS) for ten minutes; (2) monoclonal antibodies (undiluted culture supernatant or ascitic fluid diluted 1:400 to 1:1,000 in PBS-gelatine 0.2%) were overlaid on the tissue and allowed to incubate for two hours at room temperature; (3) SAM, diluted 1:100 in 0.1 mol/L Tris-HCl (pH 7.5) containing 10% normal human serum and 10% normal sheep serum, was applied to the slides. Incubation was allowed for 30 minutes at room temperature; (4) PAP complexes, diluted 1:100 in the above-mentioned Tris-HCl buffer containing 10% normal sheep serum and 10% normal human serum, were overlaid on the tissue for 30 minutes. Between steps 2, 3, 4, and 5, the sections were washed in PBS for five minutes. To improve the sensitivity of the immunoperoxidase staining procedure, steps 3 and 4 were repeated twice before (5) peroxidase activity was detected using the substrate 3-3'-diaminobenzidine (DAB) (Sigma Chemical Co, St Louis) (1

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mg DAB/mL in PBS containing 0.01% hydrogen peroxidase). After five to ten minutes' incubation in the dark, the sections were washed in PBS, dehydrated, and mounted with Malinol (Chroma, Stuttgart, FRG). Similar immunostaining results were obtained when, in steps 3 and 4 of the immunostaining procedure, normal serum was substituted by serum obtained from a patient with severe von Willebrand's disease. The possibility that all immunostaining results obtained with the monoclonal antibodies to FVIII-vWF were due to Fc-binding of these antibodies to the stained cells was excluded by the absence of reactivity with monoclonal antibodies directed to unrelated antigens of the same immunoglobulin subclasses and by the finding that saturation of Fc receptors by preincubation with aggregated gamma globulins[19] had no effect on the immunostaining results obtained with the monoclonal antibodies to FVIII-vWF. The monoclonal antibodies to unrelated antigens used were a monoclonal antibody directed to T lymphocytes (IgG_{2a}) (Becton Dickinson, Antwerp, Belgium), to mouse thymocytes (IgG_{2a}) (kindly provided by Dr W.v. Ewijk, Erasmus University, Rotterdam, The Netherlands), to trinitrophenyl (IgG_1), to house dust mite (IgG_1), and to cat allergen (IgG_1). The latter three monoclonal antibodies were kindly provided by Dr R.C. Aalberse, CLB, Amsterdam, The Netherlands.

Tissues. Tissues (pancreas, intestines, liver, kidney, lymph node, lung, heart, thymus, vena cava, and aorta) were collected at autopsies on patients deceased less than three hours. Tissue that showed no abnormality was selected for immunohistological study. Tissue of liver, kidney, and lymph node were also obtained through biopsy. Spleens were obtained from patients during surgery. Small tissue blocks were quick frozen and stored in liquid nitrogen. Bone marrow was aspirated by sternum punction, and cytocentrifuge smears were made before immunoperoxidase staining.

Cultured endothelial cells. Endothelial cells isolated from human umbilical veins were cultured[20] until confluent monolayers were obtained. After removal of culture medium and washing with PBS, the endothelial cells were incubated with RPMI 1640 (GIBCO, Biocult, UK) supplemented with 1% (wt/vol) human serum albumin. After 48 hours' incubation, samples were taken from the supernatant medium and stored at −70 °C until assayed for VIIICAg and VIIIRAg. For immunohistological studies, endothelial cells were cultured on glass coverslips until confluency was reached. After several washes with PBS to remove traces serum, cells were fixed in acetone for ten minutes and washed with PBS before immunoperoxidase staining.

Determination of VIIICAg and VIIIRAg. VIIICAg was measured using an immunoradiometric assay relying on Sepharose-linked monoclonal antibodies and human antibodies to VIIICAg.[13,14] The lower limit of detection with this assay is 0.0005 units of VIIICAg per milliliter. Determination of VIIIRAg was essentially as described by Ruggeri et al.[21] One unit of VIIICAg and VIIIRAg is the amount of antigen present in 1 mL of pooled normal plasma.

RESULTS

Localization of factor VIII (VIIICAg) in liver tissue. The localization of VIIICAg in liver biopsy material from 20 patients with mild or no detectable liver disease was investigated with an immunoperoxidase staining procedure using eight monoclonal antibodies directed against different epitopes on factor VIII. With each of these monoclonal antibodies, the most consistent finding was labeling of the cells lining the hepatic sinusoidal spaces (Figs 1 and 2). Diffuse as well as granular deposition of the immunoperoxidase reaction product could be observed in the cytoplasm of these cells (Fig 3). Hepatic parenchymal cells, as well as the vascular endothelial cells lining hepatic arteries and veins, were negative. No subendothelial deposition of immunoreactive material around arteries and veins was observed. Similarly, when liver tissue obtained from five patients with severe hepatic parenchymal disease (severe cirrhosis) was tested with these antibodies, it appeared that the cells lining the liver sinusoids were positive (results not shown). The observed immunostaining patterns obtained with these eight anti-VIIICAg monoclonal antibodies were similar, and only slight differences in staining intensity were occasionally present. Labeling of the sinusoidal liver cells could only be demonstrated by repeating the incubation cycles in the immunostaining procedure (see Materials and Methods). Apparently, one single incubation cycle in the immunostaining procedure was not sufficient to visualize the immunoreactive material present in these cells; therefore, in all subse-

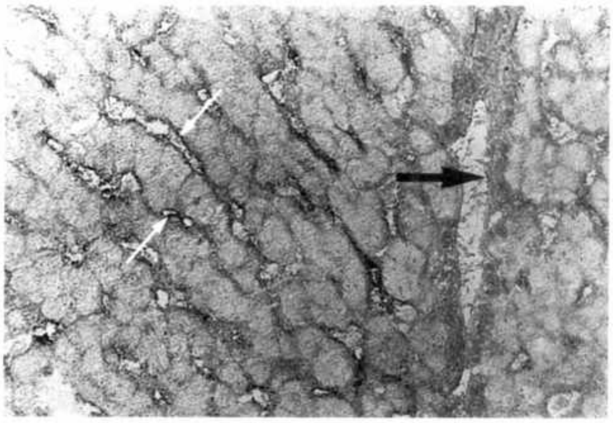

Fig 1. **Immunoperoxidase staining with anti-VIIICAg monoclonal antibody CLB-CAg 117. Section of liver tissue showing staining of the endothelial cells lining the hepatic sinusoidal spaces (white arrow). The vascular endothelial cells (black arrow) are negative (original magnification ×125; current magnification ×81; multistep immunostaining procedure, see Materials and Methods).**

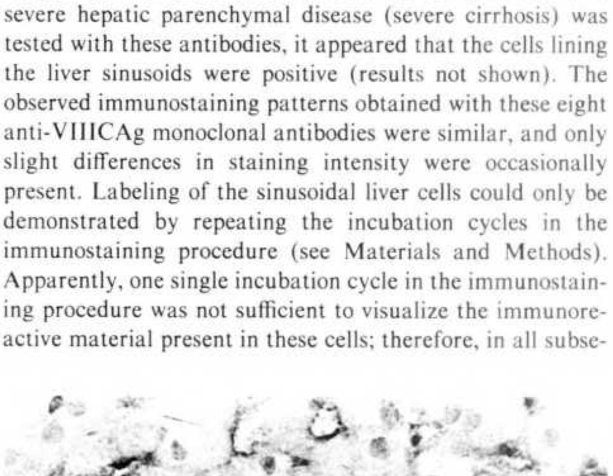

Fig 2. **Liver section stained with anti-VIIICAg monoclonal antibody CLB-CAg A. Counterstained with hematoxylin. The positive staining is limited to the endothelial cells of the sinusoids (arrow); no staining is present in the hepatocytes (original magnification ×250; current magnification ×162; multistep immunostaining procedure). h, nucleus of hepatocyte.**

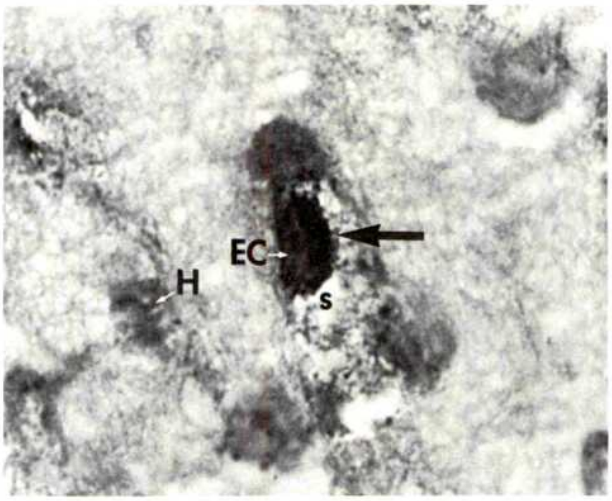

Fig 3. Liver section stained with anti-VIIICAg monoclonal antibody CLB-CAg A. Endothelial cell (arrow) lining the sinusoid shows strong granular cytoplasmatic staining (counterstained with hematoxylin). (Original magnification ×1,000; current magnification ×790; multistep immunostaining procedure.) S, sinusoid; EC, nucleus of sinusoidal endothelial cell; H, nucleus of hepatocyte.

quent experiments, we used the multistep immunostaining procedure.

In control experiments, monoclonal antibodies directed to unrelated antigens of the same subclasses as the monoclonal antibodies to VIIICAg tested were negative (Fig 4).

Localization of vWF (VIIIRAg) in liver tissue. When monoclonal antibodies to VIIIRAg were tested on liver biopsy material from 25 patients with absent, mild, or severe hepatic disease, deposition of the immunoperoxidase reaction product, both diffuse and granular, was present in the endothelial cells lining hepatic arteries and veins, as well as in the sinusoidal liver cells (Fig 5). Subendothelial deposition of immunoreactive material was noted around hepatic arteries and veins. The sinusoidal staining pattern obtained with these anti-VIIIRAg antibodies was similar to that obtained with the anti-VIIICAg antibodies (compare Figs 1 and 5). As in the case of VIIICAg, the presence of VIIIRAg in the sinusoidal liver cells could only be consistently demonstrated after repetition of the incubation cycles in the immunostaining procedure. In contrast, the presence of VIIIRAg in vascular endothelial cells was readily detectable after only one cycle of immunostaining. The most likely explanation is that this reflects differences in the amount of antigen present in these cells.

Identification of Kupffer cells in liver sinusoids. An antibody directed to lysozyme (Dako, Glostrup, Denmark) was used to identify the Kupffer cells present in the liver sinusoids. As shown in Fig 6, the Kupffer cells represent a minority of the sinusoidal liver cell population, most of these cells being sinusoidal endothelial cells.[22,23]

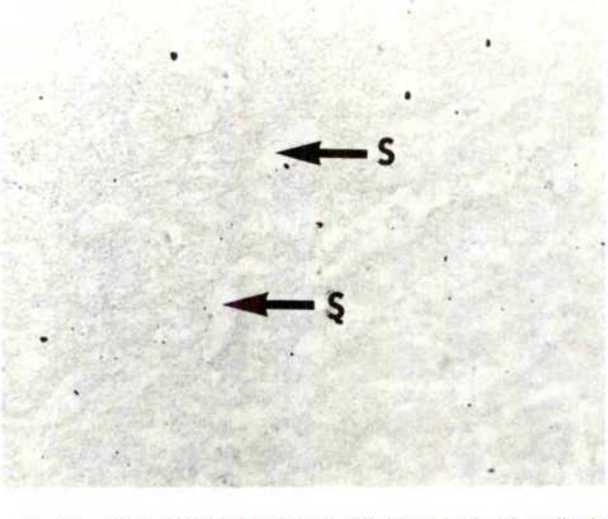

Fig 4. Liver section incubated with a monoclonal antibody directed against trinitrophenyl. No immunostaining is present (original magnification ×250; current magnification ×212; multistep immunostaining procedure). S, sinusoid.

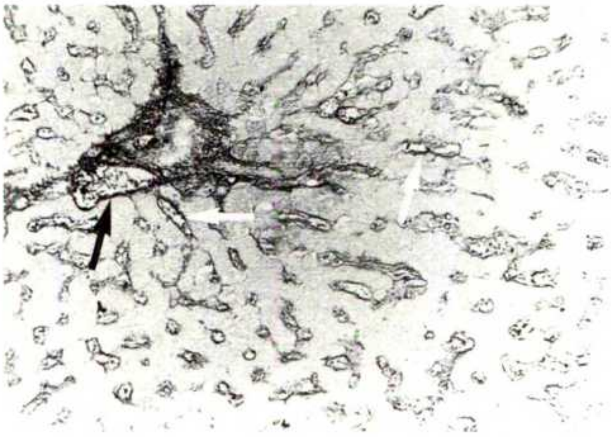

Fig 5. Immunoperoxidase staining with anti-VIIIRAg monoclonal antibody CLB-RAg 35. Section of liver tissue showing staining of sinusoidal endothelial cells (white arrow) and vascular endothelial cells (black arrow). (Original magnification ×125; current magnification ×81; multistep immunostaining procedure.)

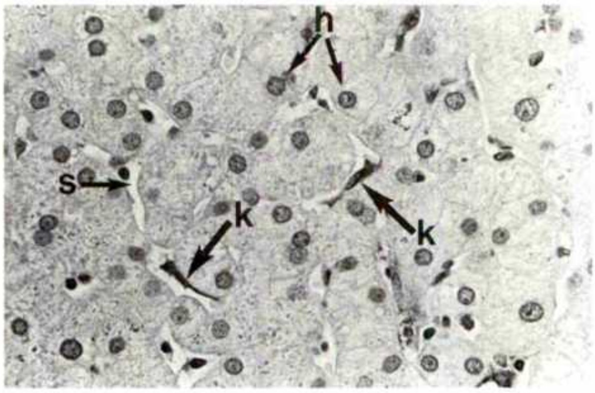

Fig 6. Immunoperoxidase staining with an antibody against lysozyme. Section of liver showing staining of Kupffer cells (K) lining the hepatic sinusoids. Counterstained with hematoxylin (original magnification ×250; current magnification ×162; one-step immunostaining procedure). h, nucleus of hepatocyte; S, sinusoid.

Localization of factor VIII and vWF in other organs. Vascular endothelial cells present in other organs (lung, spleen, lymph nodes, pancreas, intestines, heart, thymus) and the endothelial cells lining the larger blood vessels (aorta, vena cava, endocard) did not stain with any of the monoclonal antibodies to VIIICAg tested. Occasionally, a faint, scattered staining in the glomerulus of the kidney was observed; however, we were not able to confirm this unequivocally despite the recent detection of factor VIII-mRNA in renal tissue.[24] No VIIICAg could be detected in the sinusoidal endothelial cells lining the venous sinuses in the red pulp of the spleen; however, immunostaining was noticed in mononuclear cells sporadically present in the red pulp of the spleen (Fig 7), the alveolar septa of the lungs (not shown), and in lymph nodes (Fig 8). These cells, consistently found in these tissues, were stained by all eight monoclonal antibodies to VIIICAg. It appeared that the immunostaining was weak and diffuse, in contrast with the observed immunostaining in the cells lining the liver sinusoids. As deduced from the relatively large cell size and small nucleus/cytoplasm ratio, these mononuclear cells are presumably nonlymphoid and part of the mononuclear phagocyte system (probably macrophages).[25]

Positive immunostaining for VIIIRAg was present in the cytoplasm of endothelial cells lining large and small arteries, veins, and capillaries. The endothelial cells lining the renal glomeruli and lung capillaries and the cells lining the venous sinuses in the spleen were also positive (Fig 9). Staining was both diffuse and granular, and this specific staining was achieved with all four of the tested monoclonal antibodies to VIIIRAg. Subendothelial deposition of VIIIRAg could only be discerned in arteries, arterioles, and larger veins. The multistep immunostaining procedure was essential to obtain consistent positive results for the cells lining the venous sinuses in the spleen and for the glomerular endothelial cells present in the kidney. By contrast, neither in the cells lining the lymphatic vessels nor in the cells lining the lymph node sinuses could VIIIRAg be demonstrated. In addition, repeated testing with the anti-VIIIRAg antibodies of lung, lymph node, and spleen tissue failed to reveal staining for VIIIRAg in mononuclear cells present in these tissues.

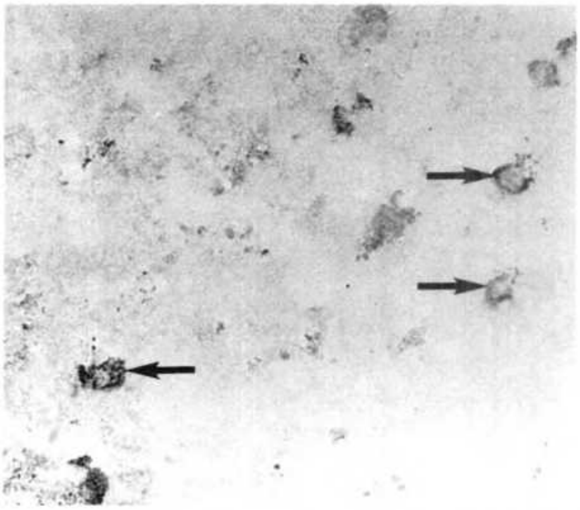

Fig 8. **Section of lymph node (paracortical area) stained with anti-VIIICAg monoclonal antibody C6. Note the labeling of mononuclear cells (arrow). (Original magnification ×125; current magnification ×112; multistep immunostaining procedure.)**

Bone marrow. None of the monoclonal antibodies to VIIICAg reacted with cells present in bone marrow. Megakaryocytes and platelets, however, showed immunostaining with all monoclonal antibodies to VIIIRAg that were tested (results not shown).

Cultured endothelial cells. The immunostaining experiments were repeated on cultured endothelial cells derived from human umbilical veins. In contrast with the monoclonal antibodies to VIIIRAg, which showed a diffuse and granular staining, the monoclonal antibodies to VIIICAg did not react with these cells. In addition, with immunoradiometric assays[13,14] capable of detecting an amount of (serum) VIIICAg as low as 0.0005 U/mL, no VIIICAg could be demonstrated in the culture supernatant, although amounts of

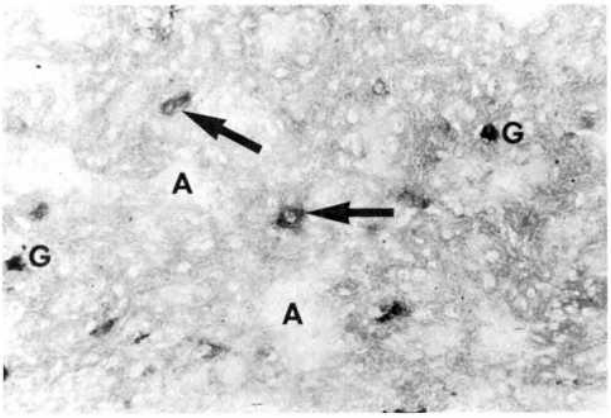

Fig 7. **Section of spleen (red pulp) stained with anti-VIIICAg monoclonal antibody CLB-CAg 58. Note the labeling of mononuclear cells (arrow) (original magnification ×125; current magnification ×81; multistep immunostaining procedure). A, spleen sinus; G, granulocyte.**

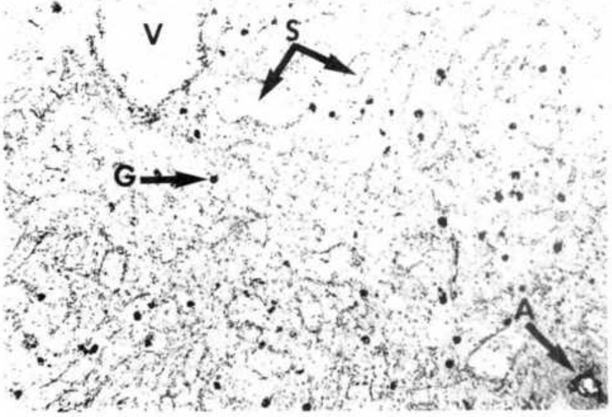

Fig 9. **Section of spleen (red pulp) stained with anti-VIIIRAg monoclonal antibody CLB-RAg 1. The cells lining the red pulp sinuses (S) and the vascular endothelial cells show deposition of the peroxidase reaction product (original magnification ×50; current magnification ×32; multistep immunostaining procedure). V, splenic vein; A, splenic arteriole; G, granulocyte.**

VIIIRAg were readily detectable (25 mU VIIIRAg/10^5 endothelial cells per 48 hours).

DISCUSSION

Using a sensitive immunostaining technique and monoclonal antibodies with different specificity against FVIII-vWF, several organs from the human body have been screened for the presence of factor VIII and vWF. The presence of material immunoreactive with anti-VIIICAg monoclonal antibodies, and hence representing VIIICAg, could be demonstrated in at least two cell types: (1) in cells lining the hepatic sinusoidal spaces, thus confirming results reported previously with only one monoclonal antibody to VIIICAg,[2] and (2) in mononuclear nonlymphoid cells occurring sporadically in lung, spleen, and lymph nodes. The latter results are corroborated by the work of Exner et al,[26] who reported a relatively high concentration of VIIICAg in lymph nodes and lung. Of those cells that were positive for factor VIII, the liver sinusoidal cells contained both VIIICAg and VIIIRAg, whereas in the mononuclear cells, only VIIICAg seemed to be present. The identity of the latter cells could not be unequivocally determined. Judging by morphological criteria (size of cell and nucleus), we tentatively propose a subset of macrophages as the most likely candidate. With regard to the identity of the VIIICAg containing liver sinusoidal cells, both Kupffer cells and sinusoidal cells can be considered as candidates. In view of the differences in staining patterns obtained with anti-VIIICAg antibodies (Fig 2) and with antilysozyme antibodies (Fig 6) (the latter being a marker for Kupffer cells), it seems likely that the presence of VIIICAg in liver is not restricted to Kupffer cells. The staining pattern obtained with the anti-VIIICAg and anti-VIIIRAg antibodies is more characteristic for hepatic sinusoidal endothelial cells, which outnumber the more patchily distributed Kupffer cells in the liver sinusoids.[23] Although the involvement of Kupffer cells cannot be excluded, the hepatic sinusoidal endothelial cells are the most likely candidates to contain both VIIICAg and VIIIRAg. In the case of VIIIRAg, this view is supported by data reported by Soda and Tavassoli.[27] Using an indirect immunofluorescence technique in combination with a polyclonal rabbit anti-vWF serum, they showed that in liver suspension of rat Kupffer cells and sinusoidal endothelial cells, only the sinusoidal endothelial cells contained VIIIRAg.

Based on the combined results of the present immunohistological study and previous recombinant DNA studies,[3-5] we favor the concept that hepatic sinusoidal endothelial cells provide a site of synthesis for factor VIII.

The possibility that factor VIII is synthesized by hepatic parenchymal cells[28] and, subsequently, stored in sinusoidal endothelial cells seems unlikely to us, since patients in the terminal stage of hepatic parenchymal disease have normal or elevated levels of factor VIII and reduced levels of vitamin K-dependent clotting factors,[29,30] the latter known to be synthesized by liver parenchymal cells.[7] The results of the present study, which show no deviate sinusoidal immunostaining with anti-VIIICAg antibodies in patients with severe hepatic parenchymal disease, are consistent with these findings.

The significance of the presence of factor VIII in mononuclear cells (probably macrophages) occurring in spleen, lung, and lymph nodes is unclear. Macrophages, with their strong phagocytic and degrading capacities, are potential sites of clearance of factor VIII. On the other hand, macrophages have been frequently reported to actively synthesize a remarkable variety of substances,[31] including clotting factors.[32-35] In this respect, it is interesting to note that these mononuclear cells reacted with anti-factor VIII monoclonal antibody C6, which does not react with serum (degraded) VIIICAg. Although this indicates that at least part of the factor VIII molecules are present in their native form, the possibility of uptake of native factor VIII cannot be ruled out. It will be clear that the issue synthesis *v* absorption cannot be resolved with immunohistological studies alone.

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Lymphokine Induction of NK-Like Cytotoxicity in T Cells From B-CLL

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Manuel O. de Landázuri, and Alberto Durántez

T cells from patients with B cell chronic lymphocytic leukemia (B-CLL) exhibit defective natural killer (NK) activity. In this study, we have analyzed the cytotoxic-inducer effects of gamma interferon (γ-IFN) and supernatants containing interleukin 2 (IL 2 sup). T cells from patients with B-CLL were incubated with γ-IFN or IL 2 sup. γ-IFN did not modulate the very low or undetectable levels of NK activity present in the T cell population. However, the IL 2 sup induced a potent cellular cytotoxicity against NK-sensitive and NK-resistant tumoral target cells. This cytotoxic inducer effect (a) was present in lectin-free IL 2 sup and in a 15,000- to 20,000-dalton molecular weight fraction obtained by gel filtration chromatography of this supernatant; (b) was directed against NK-sensitive and NK-resistant target cells; (c) was not correlated with the basal levels of NK activity; and (d) was not associated with a development or augmentation of the proportion of lymphocytes with classic NK cell phenotype. Taken together, these results demonstrate that unstimulated T cells from B-CLL patients, incubated briefly (18 hours) with IL 2 sup but not γ-IFN, have strong NK-like cytotoxicity, despite the lack of classic NK activity.

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B CELL CHRONIC lymphocytic leukemia (B-CLL) is characterized by the monoclonal expansion and accumulation of B cells, which are arrested in their differentiation pathway at an early stage.[1] There are indications that not only B cells are affected in B-CLL, but that T cells from these patients also show functional and phenotypic abnormalities.[2,3] In addition, it has been shown that E rosette-positive cells from B-CLL patients mediate defective natural killer (NK) cytotoxicity and antibody-dependent cellular cytotoxicity.[4,5] The defective NK activity present in these patients may play a role in the development of this disease, since NK activity is thought to be involved in the surveillance against tumors and in the control of both normal hematopoiesis and viral infection.[6,7]

It has been demonstrated that classic NK cells, defined by their ability to lyse a number of tumor cell line targets without prior activation, are modulated by interferon (IFN) and interleukin 2 (IL 2).[8,9] There are also reports describing the induction of killer cells[10,11] that lyse tumor cell line targets during long-term culture with lymphokine-conditioned medium. This study examined the in vitro effect of IL 2 supernatants (IL 2 sup) on T cells from B-CLL patients in the induction of cytotoxic activity against tumor target cells. Our results demonstrate that short-term (18 hours) incubation with IL 2 sup can induce strong NK-like activity in unstimulated T cells from B-CLL patients that lack classical NK activity.

MATERIALS AND METHODS

Patient population. Twenty-six previously untreated patients with clinically, histologically, and immunologically defined B-CLL (according to the Chronic Leukemia Myeloma Task Force 1973)[12] were studied. Their median age was 68 ± 9. Controls were 14 healthy, age- and sex-matched individuals.

Culture medium. RPMI 1640 (Microbiological Assoc, Walkersville, Md) supplemented with 1% L-glutamine (Flow Lab, Irvine, UK), 0.5% HEPES (Flow Lab), and 1% penicillin streptomycin (Difco Lab, Detroit) was used for cultures. This will be referred to as complete medium.

Isolation of lymphoid cells. Peripheral blood mononuclear cells (PBMs) were obtained from the heparinized venous blood of the subjects by Ficoll-Hypaque gradient centrifugation. T lymphocytes were separated by their capacity to form stable rosettes with neuraminidase-treated sheep red blood cells (SRBCs). Briefly, E-SRBCs (T cells) were obtained after incubation of PBMs with neuraminidase-treated SRBCs for two hours at 4 °C and centrifugation on a Ficoll-Hypaque gradient. The interphase, containing mainly B cells, null cells, and macrophages, is referred to as non-T cells. A T cell purity of 90% to 94% (51% ± 6% OKT4$^+$, 41% ± 10% OKT8$^+$) in the control group and 78% to 94% (36% ± 5% OKT4$^+$, 34% ± 4% OKT8$^+$) in the B-CLL group was obtained after subjecting pellets to hypotonic lysis in order to eliminate SRBCs.

Production of IL 2 supernatants. IL 2 sups were obtained from human tonsil lymphocytes stimulated with 1% phytohemagglutinin (PHA) (Difco Lab) for 24 hours, as previously described.[13] The culture supernatants were concentrated by double-step precipitation with $(NH_4)_2SO_4$ and dialyzed against phosphate-buffered saline (PBS), pH 7.4. The remaining PHA was removed by chicken red blood cell absorption, as reported elsewhere.[14] Partially purified IL 2 was obtained by gel filtration chromatography.[15] The IL 2 activity was recovered in fractions corresponding to a molecular weight (mol wt) of 15,000 to 20,000 daltons.

IL 2 was also obtained from a tumor T cell line. The Gibbon lymphosarcoma cell line, MLA-144, provided by Dr H. Rabin (Frederick Cancer Research Center, Frederick, Md), spontaneously releases a factor that is biologically and biochemically similar to human IL 2.[16] MLA-144 cells were cultured at 2 × 10^6 cells per milliliter in complete medium without serum for 48 hours. After the culture period, supernatants were collected and concentrated as previously described.

To study the production of IL 2 by the T cells from either patients or controls, 5 × 10^6 cells from each subject and control were resuspended in 1 mL of complete medium supplemented with 1% fetal bovine serum (FBS) and cultured for 24 h in the presence of 1% PHA in round-bottom culture tubes (Falcon, Oxnard, Calif). At the end of the culture period, the supernatants were collected and stored at −20 °C until tested. IL 2 assays were performed as previously described.[17]

PHA-stimulated cultures. Purified T cells were cultured in 200 μL of complete medium with 10% FBS and 1% PHA, at a density of

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1.5 × 10^5 cells per well, in triplicates in 96-well plates (Dynatech, Billingshurst, UK) for three days. Plates were incubated at 37 °C in a 5% CO_2 humid atmosphere. On day 2, 1 μ Ci of [^{3}H]-thymidine was added, and 24 hours later, cultures were harvested (Skatron, A.S., Lyerbyen, Norway) and counted in a liquid scintillation counter.

Cell cultures with IL 2 sup. Cell cultures were set up in macrowells (1.6 × 10^6 cells per milliliter) in complete medium supplemented with 10% FBS and in the presence or absence of different concentrations of IL 2 sup. The cultures were carried out for variable periods.

Cells cultured with γ-IFN. As previously described, effector cell populations were cultured in macrowells (1.6 × 10^6 cells per milliliter) in complete medium supplemented with 10% FBS for 18 hours. In the last two hours, we added 1,000 IU/mL of human γ-IFN (specific activity 2 × 10^7 IU/mg protein HEM) (Research Inc, Rockville, Md).

Cytotoxicity assays. Cytotoxicity was quantified by a ^{51}Cr-specific release assay using the following tumor target cells: K-562, Chang, MT-1, Alab, Raji, Molt-4, Molt-4-Thioguanin resistant, and Jurkat. Assays were performed at effector-target ratios of 50, 25, and 12:1 in 96-microwell, round-bottom plates. After a four-incubation, 100 μL of supernatant from each well was collected and analyzed in a gamma counter. The specific lysis (SL) and lytic units (LU) were calculated as previously described.[18]

Cell surface markers. Indirect immunofluorescence (IIF) with the monoclonal antibodies (OKT4, OKT8 [Ortho, Raritan, NJ], and Leu 11b [Becton Dickinson, Mountain View, Calif] was performed as described.[19] Samples were prepared as described for IIF[19] and analyzed with an EPICS-C Flow Cytometer (Coulter Electronics, Hialeah, Fla).

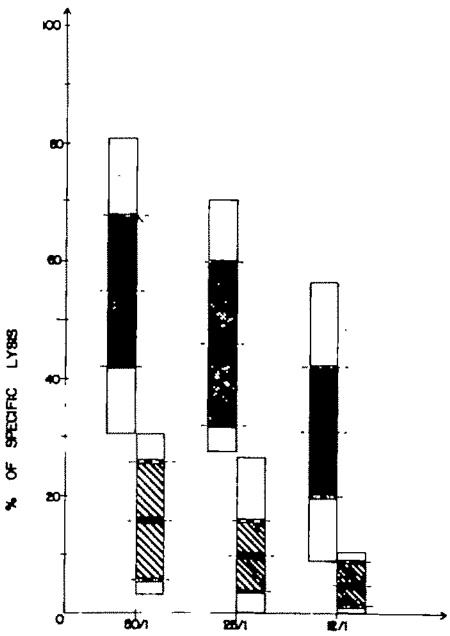

Fig 1. NK activity of T cells from B-CLL patients and normal controls at different effector-target cell ratio. The thick horizontal segments represent the geometric medium of each population. The shaded areas represent two standard deviations of the mean of each population. The extremes of the rectangles represent the rank of distribution of the data for each population. K-562 cells were used as target cells. ▨, Controls; ▨, B-CLL.

RESULTS

NK activity of PBMs, T, and non-T cells. PBMs, T, and non-T cells from 26 patients with untreated B-CLL and from 13 normal controls were separated to study their NK activity. As can be observed in Fig 1, E rosette-positive cells from B-CLL showed a significantly depressed NK activity when compared with normal controls ($P < .0001$, Mann-Whitney U test). Neither PBMs nor non-T cells from B-CLL mediated any significant cytotoxic activity (data not shown). The lack of NK activity present in the T cell-enriched population was not due to inhibition by the small number of contaminating non-T cells (range, 6% to 22%), since we observed in mixing experiments that non-T cells from B-CLL patients did not inhibit the cytotoxic activity of normal T cells (data not shown).

Modulation of the cytotoxic activity against K-562 target cells by γ-IFN and IL 2 sup. In preliminary experiments, we investigated whether IL 2 sup could induce cytotoxic activity in T cells from B-CLL patients. To this end, the T cells were incubated for two or 18 hours with IL 2 sup, and subsequently, their cytotoxic activity was tested against K-562 target cells. This revealed an increase in their lytic capacity that was maximal after incubation for 18 hours (lytic units at time 0 < 4 LU × 10^{-6} cells; after two hours – 20 LU × 10^{-6}; and after 18 hours, 40 LU × 10^{-6}). Therefore, in the following experiments, this period of incubation was routinely used.

To compare the cytotoxic-inducer effect of IL 2 with respect to γ-IFN, T cells from 12 cases of B-CLL with very low or absent levels of NK activity and T cells from controls with normal NK activity were incubated for 18 hours with these lymphokines (Table 1). After incubation with 1,000 IU/mL of γ-IFN, there was a significant increase in the cytotoxic activity of T cells in all the normal controls ($P < .05$, Wilcoxon signed-ranked test). In contrast, T cells from B-CLL patients were either unresponsive to γ-IFN (patients I, II, IV, and VI) or minimally so (rest of the patients). When the data of all 12 patients were analyzed together, the increments observed in NK activity after γ-IFN treatment were not statistically significant ($P > .05$, Wilcoxon signed-ranked test). On the other hand, when all the data from either patients or controls were examined, we did observe a significant correlation ($P < .05$, least squares method) between the levels of cytotoxicity observed after γ-IFN treatment and the basal values of NK activity (Fig 2).

The incubation with IL 2 sup of T cell from each of the B-CLL patients induced a significant increase in their ability to lyse K-562 target cells, which proved significant when all the cases were analyzed together ($P < .01$, Wilcoxon signed-ranked test). We also observed that the highest levels of cytotoxicity induced by IL 2 sup were obtained precisely in those patients who did not show spontaneous cytotoxic activity and did not respond to γ-IFN. We did not observe any correlation, either in B-CLL patients or in normal controls, between the basal levels of NK activity and the killing activity after incubation with IL 2 sup (Fig 2).

Table 1. Effects of γ-IFN and IL 2 Sup on Cytotoxic Activity of T Cells From B-CLL Against K-562 Target Cells

	Specific Lysis								
	Basal			After Treatment With γ-IFN			After Treatment With IL 2 Sup		
Subjects Patients	50/1	25/1	12/1	50/1	25/1	12/1	50/1	25/1	12/1
I	13	3	0	14	9	1	35	14	5
II	12	8	5	24	18	13	34	24	16
III	10	5	2	7	3	0	70	60	49
IV	5	2	0	6	4	0	70	50	26
V	3	0	0	2	0	0	47	34	19
VI	3	1	0	4	2	1	76	60	46
VII	4	3	0	14	3	1	35	14	9
VIII	19	12	0	44	21	11	49	35	15
IX	13	9	1	19	13	11	43	23	19
X	17	9	4	29	22	16	51	38	26
XI	14	10	6	34	27	14	49	42	23
XII	19	16	11	30	22	15	50	42	31
Patients (x̄ ± s)	11 ± 6	6 ± 4	2 ± 3	18 ± 13	12 ± 9	6 ± 6	50 ± 14	36 ± 15	23 ± 13
Controls (x̄ ± s)	45 ± 18	36 ± 15	25 ± 17	72 ± 15	61 ± 9	45 ± 22	76 ± 20	66 ± 25	51 ± 26

T cells from 12 B-CLL patients and six normal controls were incubated for 18 hours either with complete medium; complete medium for the first 16 hours, γ-IFN (1,000 IU/mL) being added in the last two hours; or complete medium plus 20% IL 2 sup. After this period of time, the cultures were analyzed for cytotoxic activity against K-562 target cells. The data are given as the percentage of specific lysis at effector-target cell ratios of 50:1, 25:1, and 12:1.

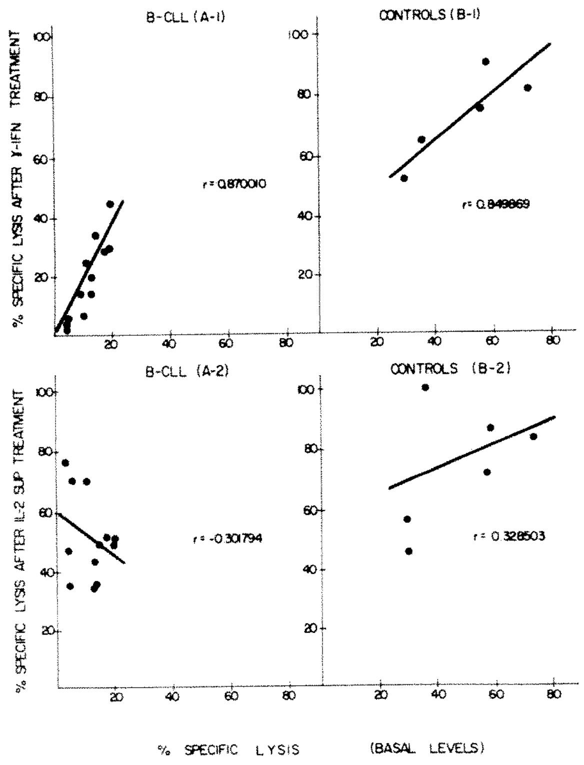

Fig 2. Correlations between the basal levels of cytotoxicity and (A) the lytic activity of T cells from B-CLL after incubation with γ-IFN (A-1) or IL 2 sup (A-2) and (B) the lytic activity of T cells from controls after incubation with γ-IFN (B-1) of IL 2 sup (B-2). The cytotoxic activity is expressed as a percentage of specific lysis against K-562 target cells at an effector-target cell ratio of 50:1.

This finding led us to study the possibility that the T cells from B-CLL patients could be deficient in their synthesis of IL 2. We found that IL 2 production by PHA-stimulated T cells from nine patients were similar to that of six normal controls (Fig 3). However, the proliferative response to PHA stimulation of the T cells from these B-CLL patients

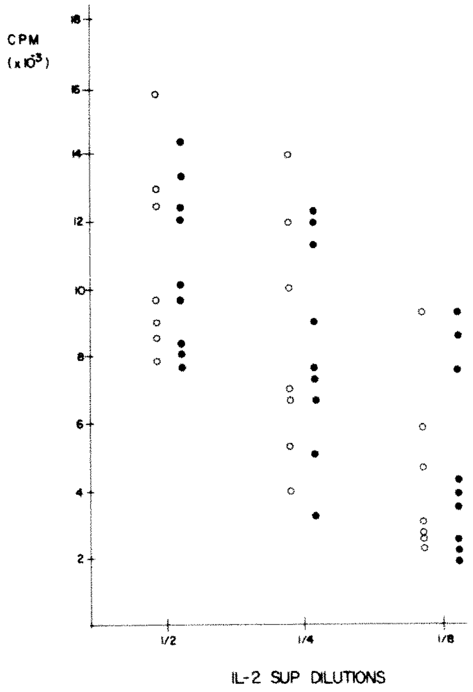

Fig 3. Proliferative response of long-term cultured human T cells after a 36-hour incubation period, in culture, with different proportions of IL 2 sup produced by T cells from either B-CLL patients (●) or normal controls (○).

(34,407 ± 15,709 cpm/1.5 × 10^5 cells) was significantly decreased ($P < .01$, Mann-Whitney U test) when compared with that of the normal controls (87,082 ± 21,179 cpm/1.5 × 10^5 cells).

We also ruled out the possibility that the enhancement of cytotoxic activity by IL 2 sup was due to activation of the contaminating non-T cells, since we did not observe any cytotoxic activity when non-T cells from B-CLL patients were incubated with IL 2 sup (data not shown). To investigate whether the observed effects were due to PHA that could still be present in the IL 2 sup, we used supernatants from the T cell line, MLA-144, which have been shown to produce IL 2 constitutively. As can be seen in one representative experiment (Fig 4), MLA-144 sup induced an increase in the cytotoxic activity of T cells from B-CLL patients similar to that of IL 2 sup. Finally, and to further delineate the molecules responsible for the enhancement of the cytotoxic activity, we used a semipurified preparation of IL 2 obtained by gel filtration chromatography (15,000 to 20,000 mol wt). As can be seen in Fig 4, this preparation also induced strong cytotoxic activity in T cells from B-CLL patients.

We investigated the target cell specificity of the T cells from B-CLL patients after incubation with IL 2 sup. As can be seen in Fig 5, short-term incubation with IL 2 sup induced lytic activity against both NK-sensitive and NK-resistant target cells.

Finally, since the previous results demonstrated that IL 2 sup induced strong NK-like activity in the T cells from B-CLL patients, we studied whether this cytotoxic-inducer effect could be explained by an increase in the percentage of lymphocytes with the classic NK cell phenotype. In five experiments we observed that incubation of the IL 2 sup did not modify the low percentage of Leu 11^+ cells (<1%) present in the T-enriched populations from the B-CLL patients.

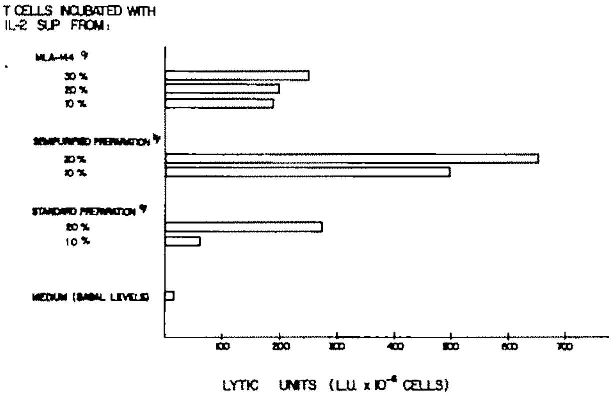

Fig 4. Cytotoxic activity of T cells from B-CLL patients after treatment with different IL 2 preparations. T cells were incubated either with complete medium (basal levels) or with the indicated concentrations of IL 2 from the T cell line MLA-144 (a), from a semipurified preparation obtained by gel chromatography (mol wt 15,000 to 20,000 daltons) (b), or from a standard preparation (c). After 18 hours of treatment, the cultures were analyzed for cytotoxic activity against K-562 target cells. The data are given in lytic units, LU 10^{-6} cells, as described in the text.

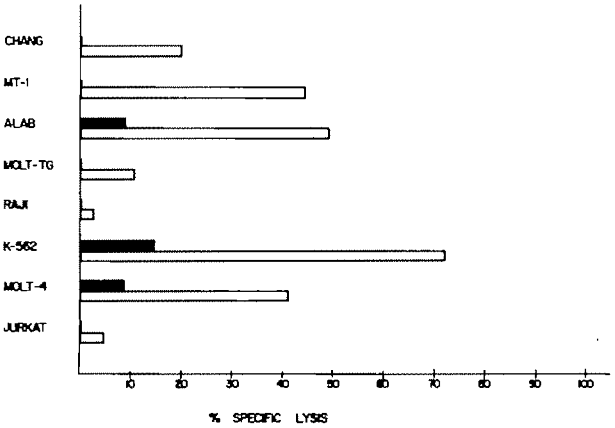

Fig 5. Cytotoxic activity of T cells from B-CLL against different target cells. T cells were incubated with either complete medium (□, closed bars) or 20% IL 2 sup (□, open bars). After 18 hours of treatment, the cultures were analyzed for cytotoxic activity against Chang, MT-1, Alab, Molt-TG, Raji, K-562, Molt-4, and Jurkat target cells. The data are given in percentage of specific lysis at an effector-target cell ratio of 50:1.

DISCUSSION

This paper, above all, poses an intriguing question regarding the nature of the IL 2 sup-induced cytotoxicity generated in the T cells obtained from untreated B-CLL patients. The origin of these effector cells may be explained by (a) the activation of already mature NK cytotoxic cells; (b) the differentiation of pre-killer NK cells into effective cytotoxic cells; or (c) the recruitment of a different cell population not related to the NK pathway. Our data suggest that this last possibility is the most likely. The following reasons support this contention. First, there was no correlation between the basal levels of NK activity and the cytotoxicity acquired after incubation with IL 2 sup. Second, mature NK cells responded to IFN with an increment in their lytic activity, but using T cells from B-CLL patients, we did not observe this enhancement. (Other authors have also reported this observation.[4,5]) Third, the cytotoxicity generated by the IL 2 sup was active against both NK-sensitive and NK-resistant target cells. Finally, there was no induction of cytotoxic effector lymphocytes with phenotype of classic NK cells after the incubation with IL 2 sup.

Furthermore, recent reports in the literature support the possibility that, in some systems, the effector cells generated by IL 2 are not typical mature NK cells. We have observed that human thymocytes cultivated in the presence of IL 2 sup acquire de novo cytotoxic activity against tumor target cells.[15] Grimm et al[10] showed that PBMs incubated with IL 2 acquire strong cytotoxic activity against NK-resistant target cells. Although the mechanisms underlying these observations remain to be determined, the effector cells in these models have several similarities to those described by us in this report. For example, these effector cells can be generated by IL 2 in populations devoid of spontaneous NK activity and have neither the specificity nor the phenotypic markers of mature NK cells. The relationship of these effector cells with classic NK cells is unknown.

Several pathological conditions in addition to B-CLL have been found to have similar immunological abnormalities, including an inverted T4/T8 ratio, a poor response to T cell mitogen, and a loss of NK activity.[20-23] In the acquired immunodeficiency syndrome, Rook et al[23] have recently shown that the depressed NK activity present in PBMs of these patients cannot be restored by γ-IFN, but can be raised to normal levels by treatment with IL 2 sup. These results are identical to those obtained by us with B-CLL patients. Thus, it is possible that the defective NK activity present in B-CLL and other diseases could be overcome by in vitro treatment with IL 2.

Further studies are needed in order to understand (a) the relationship between the effector cells mediating NK activity and those generated in the presence of IL 2; (b) the significance of the T cell abnormalities in B-CLL; and (c) the potential immunotherapeutic use of lymphokines in this group of diseases.

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Cytoskeleton Organization Is Aberrantly Rearranged in the Cells of B Chronic Lymphocytic Leukemia and Hairy Cell Leukemia

By Federico Caligaris-Cappio, Luciana Bergui, Luisa Tesio, Guelfa Corbascio, Franca Tousco, and Pier Carlo Marchisio

The organization of actin-containing microfilaments and vimentin-containing intermediate filaments has been investigated in B chronic lymphocytic leukemia (B-CLL), hairy cell leukemia (HCL), and normal B cells cultured in vitro under basal conditions and after induction with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). In uninduced B-CLL cells, F-actin was predominantly associated with dot-shaped structures scattered over the ventral membrane representing spotty close contact adhesion sites analogous to "podosomes" described in other cell types. On TPA induction, podosomes became clustered in sharply defined areas sitting in the cell center beneath the nucleus. In some cells, long actin-containing protrusions appeared. In HCL cells, F-actin was associated with thin microvilli responsible for the "hairy" appearance; occasional cells showed scattered podosomes. On TPA induction, HCL cells sprouted long dendritic processes rich in submembraneous F-actin, which made intertwined networks. Therefore, in both B-CLL and HCL cells, adhesion structures were present and the capacity for adhesion in vitro was marked, which might explain some peculiar clinical features of the diseases. Adhesion structures and adhesive properties never appeared in normal B cells. These data further support the notion that B-CLL and HCL, although clinically different, may share common biological features and suggest that in these disorders, cytoskeleton modifications may represent a hallmark of transformation.

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A NUMBER OF MAJOR lymphocyte functions mediated by surface receptors are controlled by the cytoskeleton. These functions include blood-lymph recirculation, cell-cell interactions, and secretion of antibodies and lymphokines (for review, see refs 1 and 2).

The malignant cells of B chronic lymphocytic leukemia (B-CLL) and hairy cell leukemia (HCL) are characterized by abnormalities that suggest defects of cytoskeletal functions. B-CLL cells have low mobility,[3] show decreased capping by different ligands,[4-6] and are unusually susceptible to microtubule disrupting drugs.[7,8] HCL owes its name to the presence of many irregular projections protruding from the surface of cells, which are clearly visible in a phase contrast microscope and appear as irregular undulating ruffles or long villi when examined by the scanning electron microscope.[9] HCL cells are able to cap surface immunoglobulins (sIgs) and concanavalin A (ConA) receptors; the membrane redistribution of these structures is inhibited by cytochalasin B.[10,11]

The in vitro stimulation of B-CLL and HCL cells with the differentiation-promoting agent 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induces changes of morphology, cytoplasmic enzyme activity, and surface markers.[12-14] B-CLL cells acquire an irregular hairy cell-like appearance with ruffles and protrusions.[12,13] HCL cells become firmly attached to culture dish surfaces and exhibit fibroblast-like projections and stellate features.[12-14] Details of the action of TPA are not yet fully elucidated; recent evidence suggests that TPA permeates the plasma membrane and activates protein kinase C, mimicking the physiological activator diacylglycerol.[15,16] By such mechanism, some protein targets are phosphorylated and this leads to reorganization of the subsurface microfilamentous network.[17-21]

These observations provide the rationale for the present study, in which we have investigated the organization of the cytoskeleton of normal B, B-CLL, and HCL cells in the uninduced state and after TPA induction in vitro. The experimental approach was adopted to answer the following questions: (1) Can TPA induce a rearrangement of cytoskeletal structures in malignant B cells? (2) Are the peculiar morphological features of HCL, also reproduced by TPA treatment of B-CLL cells, dependent on cytoskeleton rearrangements? (3) Is the cytoskeleton of normal B cells behaving differently compared with its malignant counterparts under the same experimental conditions?

MATERIALS AND METHODS

Preparation of cells. Peripheral blood lymphocytes (PBLs) were obtained from 26 patients. Twenty-three patients (14 males, nine females; aged 49 to 70) had B-CLL: ten were in stage O-I, six in stage II, six in stage III, and one in stage IV, according to Rai.[22] In all cases, B lymphocytes expressed monoclonal Ig (k^+ or λ^+) with low intensity, formed rosettes with mouse red blood cells ($MRBC^+$), and were Tl antigen positive as detected by the monoclonal antibody RFTl.[23] None of the patients had received chemotherapy in the four months preceding the study. Three patients (all males aged 52 to 68) had HCL. The diagnosis was based on cell morphology, the presence of 50% to 75% tartrate-resistant acid phosphatase-positive ($TRAP^+$) cells in peripheral blood, and the bone marrow histology on trephine biopsies.

Mononuclear cells were separated from patients' peripheral blood samples on Ficoll-Hypaque (FH). Cells layered at the interphase were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI medium supplemented with 10% fetal calf serum (FCS). The percentage of monocytes was 1% to 4% in B-CLL samples and less than 1% in HCL samples.

Tonsils (three samples) were obtained from children undergoing tonsillectomy after antibiotic treatment and were teased with blunt forceps. Cell suspensions were washed with PBS and allowed to adhere to plastic for one hour. The nonadherent cells were rosetted with sheep red blood cells (SRBC) and spun onto FH for 30 minutes at room temperature. The cells at the interphase were collected and resuspended in RPMI containing 10% FCS and the percentage of

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residual T cells was evaluated with Leu 4 (Becton Dickinson, Mountain View, Calif, cat. 7340). In one experiment, MRBC$^+$ cells were purified as previously detailed.[12,13] Briefly, 5×10^6/mL tonsil lymphocytes were incubated with 10^8/mL MRBCs as a pellet for five minutes at 37 °C and then for 30 minutes at 4 °C.[24] The cells, after gentle resuspension, were spun onto FH at 400 g for 25 minutes. The pelleted cells were treated with 0.1% Tris-NH_4Cl for 15 minutes at 20 °C to lyse RBCs and spun through FCS.

In vitro cultures. B-CLL, HCL, and normal B cells were resuspended in RPMI 1640 supplemented with 10% FCS at 1×10^6 cells per milliliter. Cells were cultured in vitro in 5-mL cultures and incubated for 72 hours on glass coverslips in 60-mm tissue culture dishes (Falcon, Oxnard, Calif) at 37 °C in a water-saturated atmosphere containing 5% CO_2. In each experiment, duplicate cultures were set up as controls and as TPA-stimulated samples. TPA was first dissolved in acetone (1.6 mmol/L) and then in absolute ethanol (0.016 mmol/L). The final TPA concentration in the cultures was 0.016 μmol/L, which previous experiments[12] had shown to be optimal for inducing phenotypic changes without major decrease in viability.

After 72 hours, the percentage of control uninduced B-CLL and HCL cells that showed spontaneous adherence to coverslips was 50% to 80%. The percentage of adherent TPA-induced B-CLL and HCL cells was more than 80%. Of the nonadherent cells, 10% to 20% were nonviable as assessed by trypan blue dye exclusion. On the contrary, almost all normal B cells were nonadherent, and the percentage of dead cells was 5% to 10%. The free-floating cells were harvested and glued onto poly-L-lysine–treated glass coverslips for 30 minutes. The slides were then washed briefly with PBS, allowed to dry, fixed and stained as described.

Fluorescence microscopy. Coverslip-attached cells were processed both for the expression of surface markers and for the analysis of cytoskeletal structures.

The expression of surface markers was investigated on air-dried, acetone-fixed cells stained in double fluorochrome immunofluorescence using mouse monoclonal antibodies (Mo Abs), followed by goat antimouse IgGs tagged with fluorescein isothiocyanate (FITC) and goat antihuman Ig isotypes tagged with tetraethylrhodamine isothiocyanate (TRITC). We used the B cell-specific Mo Abs B1 (Coulter cat. No. 6602140), B4 (Coulter, Hialeah, Fla, cat. NO. 6602683), RFB4[25] gift of Professor G. Janossy, London), the monocyte-reacting MO1 (Coulter, cat. No. 6602147), MO2 (Coulter cat. No. 6602141), UCHM1[26] (gift of Dr P. Beverley, London), and rabbit antisera to human Ig isotypes (μ, δ, γ, α, κ, λ) labeled with TRITC from Dakopatt (Glostrup, Denmark, cat No. R-152,R-148,R-151,R-153,R-154,R-155). MO1 and MO2 Abs are not monocyte-specific, since they react with granulocytes and bone marrow precursors.[27] Nevertheless, they are unreactive with normal and malignant B cells while detecting both normal and malignant monocytes.[28] Thus, MO1 and MO2 discriminate between B lymphocytes and cells of the monocyte-macrophage lineage.

The analysis of cytoskeletal structures was performed on coverslip-attached cells fixed in 3.7% formaldehyde (from paraformaldehyde) in PBS containing 2% sucrose for 5 minutes at room temperature. After rinsing in PBS, cells were permeabilized in PBS containing 0.5% Triton X-100 (BDH Chemicals, Ltd, Poole, England) for 5 minutes at room temperature and extensively rinsed.

F-actin–containing microfilamentous structures were stained for 30 minutes at 37 °C with rhodamine isothiocyanate-labeled phalloidin (R-PHD) dissolved in PBS containing 0.5% dimethyl sulphoxide. R-PHD, which has been shown to selectively bind to F-actin,[29] was a kind gift of Professor Th. Wieland, Heidelberg, FRG.

Vimentin-containing intermediate-type filaments were decorated by a mouse Mo Ab which has been shown to recognize vimentin of most animal species.[30] The vimentin antibody was used as hybridoma supernatant directly layered on detergent-permeabilized cells and incubated for 30 minutes at 37 °C. After rinsing with PBS, coverslips were incubated with either FITC- or TRITC-tagged rabbit antimouse IgGs (Dakopatt, cat. No. R-270) used at 0.1 mg/mL for 30 minutes at 37 °C. In some experiments, F-actin was simultaneously decorated by adding R-PHD (1 μg/mL) in the second antibody step.

Stained coverslips were mounted either in 50% glycerol in PBS or in Mowiol 4-80 (Hoechst, Frankfurt, FRG) and viewed with the appropriate filters in a Leitz Dialux epifluorescence microscope equipped with a Zeiss planapochromat 63×/1.4 oil immersion lens. For determining adhesion, PBS-glycerol–mounted coverslips were examined at 50× power in a Leitz Diavert microscope equipped for interference-reflection contrast.

Fluorescence photographs were recorded on Kodak Tri × films rated at 1250 ISO and developed in Gradual ST20 developer (Ornano, Milan). Interference reflection pictures were recorded on Agfa Ortho 25 films.

Finally, in each experiment, coverslip-attached cells were checked for the presence of nonspecific acid esterase with standard cytochemical methods.

RESULTS

Characterization of B cell populations. Normal tonsil B cells were strongly IgM$^+$ and did not form rosettes with MRBCs. The purified MRBC$^+$ cells were weakly IgM$^+$ and coexpressed the T1 antigen (RFT1$^+$). Both cell populations reacted with B1, B4, and RFB4 (95%) and did not stain with Mo1, Mo2, UCHM1 (1%). The phenotype was left essentially unchanged by TPA treatment.

B-CLL cells showed the typical features of weak surface Ig expression (80%) and MRBC positivity (60% ± 9%). In 21 of 23 cases, the monoclonal B cells were RFT1$^+$ (90%). All cases were also B1$^+$, B4$^+$, RFB4$^+$ (85%) and failed to react with Mo1, Mo2, UCHM1 (1%). After TPA, all cases lost sIg; B1, B4, and RFB4 reactivity became faint or was lost. As already shown in previous studies,[12,13] the T1 antigen expression was retained. Sixteen of 23 cases expressed cytoplasmic Ig of the same heavy and light chain expressed by fresh uninduced cells. In no instance was positivity with Mo1, Mo2, or UCHM1 recorded (less than 1%).

HCL cells were Ig$^+$, reacted with B1, B4, RFB4 (80%) but failed to stain with Mo1, Mo2, UCHM1 (5%). The phenotypic features were left essentially unchanged after TPA treatment. B-CLL and HCL cells, in both uninduced state and after TPA, never showed cytoplasmic nonspecific esterase enzyme activity.

Microfilaments in normal and TPA-induced human B lymphocytes. Substratum-attached human B lymphocytes appear as spherical cells provided with thin laminar slabs accounting for their attachment. The distribution of F-actin–containing microfilaments is difficult to document because of the rounded shape of cells, which is responsible for a uniform fluorescent signal associated with the plasma membrane (Fig 1A). After 72 hours' exposure to TPA, B cells tend to flatten on a poly-L-lysine substratum, giving rise to a polymorphic population in which F-actin is mostly associated to short peripheral protrusions. Such extensions are in the form of slender (Fig 1B) or thicker (Fig 1C) microvilli, flattened ruffles (Fig 1C and D), and thorns (Fig 1E). Occasionally, arrowhead-shaped protrusions containing

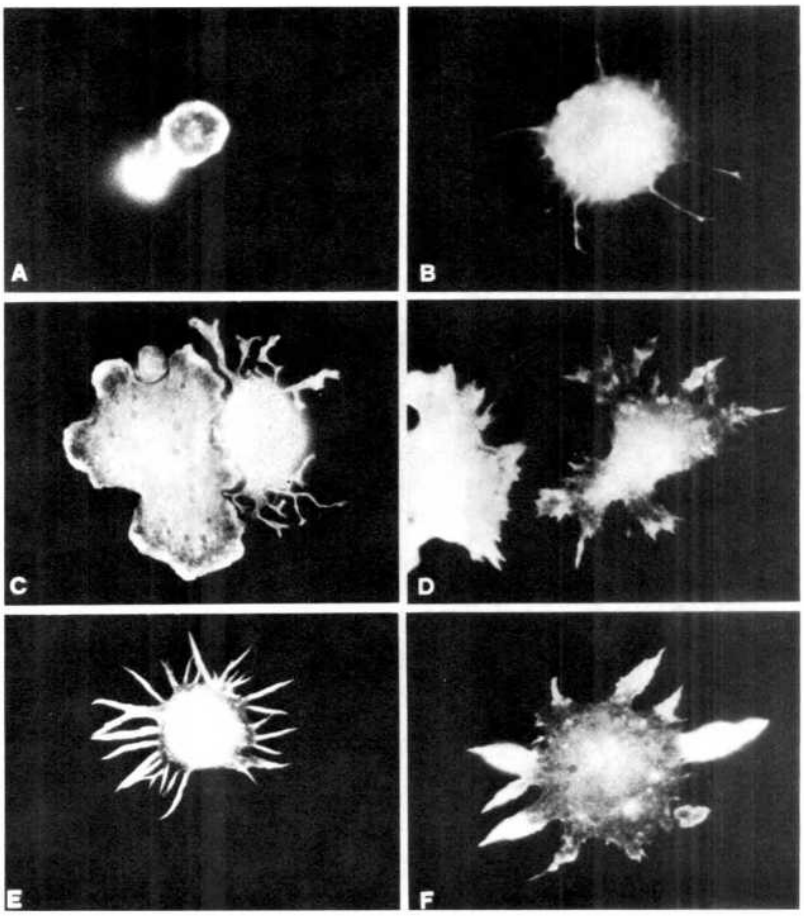

Fig 1. R-PHD fluorescence staining of F-actin in normal (A) and TPA-induced B cells (B through F). In normal B cells, F-actin is associated with the cytoplasmic face of the plasma membrane and gives rise to a thin line of peripheral fluorescence (A). After 72 hours' induction with TPA, B cells generally spread over the substratum and sprout multiple processes, showing different patterns of microfilamentous structures (original magnification ×3,500; current magnification ×2,275).

large amounts of F-actin sprout from the cell periphery (Fig 1F).

After TPA treatment, the general pattern of microfilaments suggests that B cells have arranged their actin-containing structures and have probably acquired motility properties that are not expressed before treatment. The marked polymorphism of TPA-treated B cells suggests analogy with the cytoskeletal pattern expressed by control HCL cells (see later).

Microfilaments in untreated B-CLL and HCL cells. In cultured B-CLL cells, three phenotypically variant forms can be identified. In each case examined, a small proportion (about 10%) of cells are rounded, and their appearance and staining pattern with R-PHD are barely distinguishable from control B lymphocytes (eg, Fig 1A). This cell population probably represents a variant that cannot achieve adhesion with the culture substratum. The size of this subpopulation may be underestimated because staining manipulations may have removed a considerable proportion of these cells.

The majority of cells (about 80%) shows a characteristic staining pattern with R-PHD. The main feature is the concentration of F-actin in multiple, rather regularly distributed dots that, by careful focusing, appear to be associated with the ventral membrane sitting in contact with the adhesion substratum (Fig 2A). When examined in the interference reflection microscope, which measures the distance between cells and the adhesion substratum,[31,32] these dots appear to correspond to close contact areas (Fig 2B). In these cells, the nucleus is mostly centrally located (Fig 2C). Further evidence that actin-containing dots are associated with punctate adhesion sites can be inferred from the fact that, when cells are gently streamed with a jet of buffer before fixation, dots remain attached to the substratum and leave a footprint of the cell ventral membrane.[33]

In a third subpopulation, representing a minority of attached cells (about 10%), the dots are absent, but the general shape is more irregular because polymorphic protrusions sprout from the cell surface. Such pattern is similar to that exhibited by TPA-induced normal B lymphocytes (see Fig 1B through F) and represents the main phenotype of in vitro HCL cells (see later). B-CLL cells showing mixed features, ie, both dots and processes, are observed with low frequency (less than 5%).

As a rule, the majority of control HCL cells show a wealth of protrusions in the form of filopodia and ruffles, which are located both at the peripheral rim and over the dorsal surface (Fig 3A). Such protrusions are clearly visible on fluorescent PHD-stained cells on the basis of their rich F-actin content. A minor fraction of HCL cells (around 20%; Fig 3B) show dot-like accumulations of F-actin at the ventral membrane similar to those shown by the majority of B-CLL cells and some thin microfilament bundles (Fig 3B). Cells showing both dots and membrane protrusions (eg, Fig 3C) are infrequently observed (about 5%).

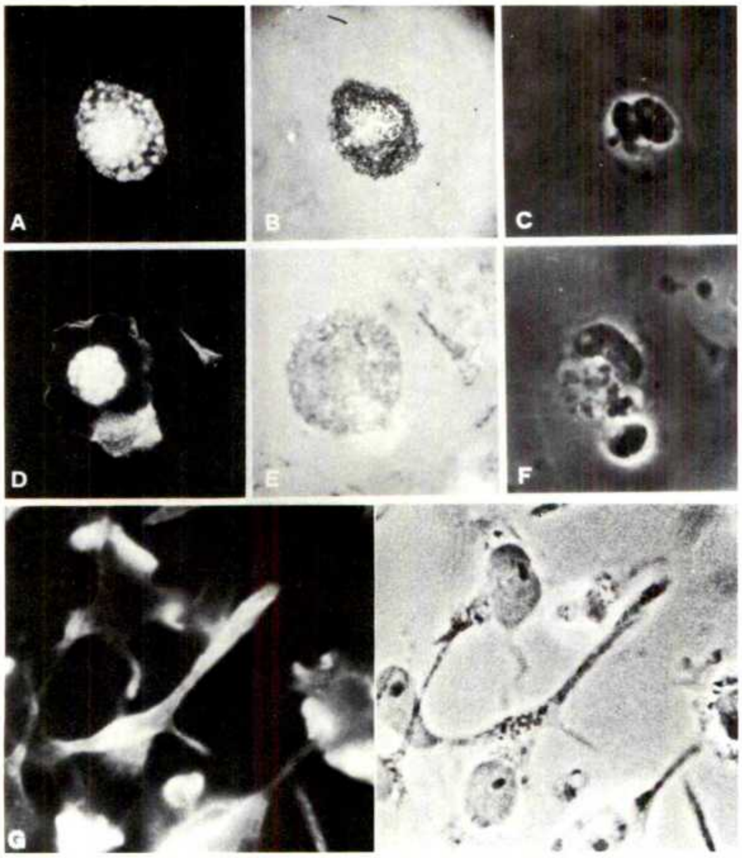

Fig 2. R-PHD fluorescence staining of F-actin in uninduced (A) and TPA-induced B-CLL cells (D, G). Interference reflection and phase contrast pictures of the same cells are shown, respectively, in (B) and (E) and in (C), (F), and (H). In uninduced B-CLL cells (A), actin-containing podosomes are scattered all over the ventral membrane and give rise to a grayish pattern of close contact in the interference reflection microscope (B). The nucleus is apparently located in the cell center (C). After TPA induction, podosomes cluster in a sharply defined area (D) that corresponds to spotty close contacts (E). The nucleus is generally displaced outward by the cluster of podosomes, suggesting that these structures extend deeper in the cytoplasm of these cells (F). A nonadherent cell overlying the flattened one is out of focus and barely visible (lower right) in (D), (E), and (F). In some induced B-CLL cells, sprouting of long dendritic processes takes place (G and H). Such a pattern is identical to that induced by TPA in HCL cells (see Fig 3). (Original magnification ×3,500; current magnification ×2,275)

Changes of microfilament pattern induced by TPA treatment in B-CLL and HCL cells. TPA treatment induces marked changes in the microfilament distribution of B-CLL and HCL cells. The most remarkable effect in B-CLL cells is the constant clustering of the dot-like accumulations of F-actin observed in untreated cells, which comes along with considerable flattening and improvement of adhesion (Fig 2D). Clusters of dots are usually positioned in the area below the nucleus but may be eccentric or clearly displace the nucleus, which sometimes acquires a bean-like shape (Fig 2F). In the interference reflection microscope, a broad close contact area appears to be coincident with the cluster of dots (Fig 2E) and often extends beyond it. The clustering of dots is a regular event on TPA treatment and can be observed in most cells in the vast majority of cases.

In a number of B-CLL samples (about 30%), TPA treat-

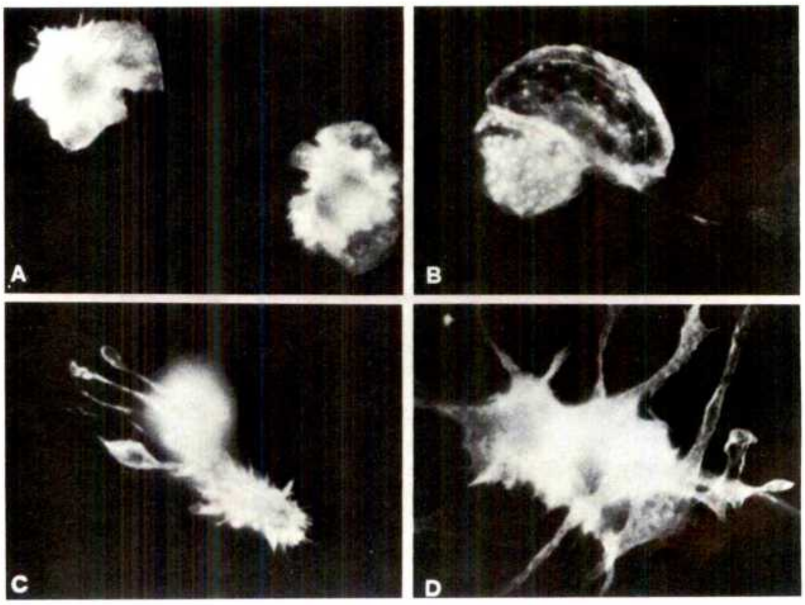

Fig 3. R-PHD fluorescence staining of uninduced (A through C) and TPA-induced (D) HCL cells. Uninduced HCL cells show a polymorphous pattern of ruffles and peripheral protrusions, justifying the "hairy" appearance. Some cells show obvious podosomes (B, lower left) and, occasionally, thin microfilament bundles (B, upper right). On TPA induction, HCL cells sprout long processes that make intertwined networks reminiscent of some tissue cultures of nerve cells (D). (Original magnification ×3,500; current magnification ×2,275)

ment induces also the appearance of polymorphic stellate cells whose long processes form a complex intertwined network (Fig 2G and H). Within such network, scattered cells with clustered dots are sometimes found together with intermediate forms, thus suggesting that cells showing either stellate appearance or clustered dots may represent a continuum of interchangeable forms (not shown).

In all three cases of HCL examined, TPA treatment induces the appearance of stellate cells forming an intricate dendritic network (Fig 3D). Microfilaments are associated with the membrane in polymorphic protrusions. The presence of clustered F-actin dots is never observed in TPA-treated HCL cells.

Distribution of vimentin-type intermediate filaments in normal B, B-CLL, and HCL cells. Substratum-attached normal B lymphocytes have intermediate filaments of the vimentin type located in the thin perinuclear cytoplasmic area (Fig 4A). After TPA treatment, along with cell flattening, intermediate filaments tend to form curly bundles extending within the cytoplasmic lamina but never reach the edge of the cell (Fig 4B). Those cells that just extend thin filopodia never show vimentin within such extensions.

In double-labeled B-CLL cells, complete independence exists between the network of vimentin filaments and the distribution of F-actin dots. Accurate focusing suggests that intermediate filaments lay in an upper layer within the cell.

Vimentin filaments form intricate networks in flattened B-CLL or HCL cells (Fig 4C) and do extend into elongated cellular processes of these cells after TPA induction (Fig 4D).

DISCUSSION

The close association between lymphocyte receptor function and the cellular contractile system has focused attention on lymphocyte cytoskeleton (for review, see refs 1 and 2). Both normal and malignant lymphoid cells have been analyzed, and several reports have detailed the biochemistry and function of cytoskeletal proteins in B-CLL and HCL.[34-38] The present study was undertaken to evaluate the organization of some cytoskeletal structures in HCL and B-CLL cells, as well as in normal B cells, and the rearrangements that might occur on activation in vitro. Our approach has been to study cultured adherent cells at rest or after activation with TPA. The rationale of this approach is threefold. First, adherent cells allow a detailed analysis of intracellular filamentous networks. Second, TPA has been shown to influence both shape and motility of different cell systems.[39] Finally, a close lineage relationship has recently been established between B-CLL and HCL by culturing the malignant cells in vitro in the presence of nanogram TPA concentrations and demonstrating that TPA-induced B-CLL cells acquire the typical morphology, enzyme activity, and surface markers of HCL cells.[12,13]

Our first observation is that circulating uninduced HCL cells already have an altered organization of the microfilament pattern, since they show a wealth of peripheral microvilli, which account for their "hairy" appearance. These are absent in both normal and other neoplastic B cells. TPA stimulation emphasizes such pattern by producing long dendritic processes in which intermediate filaments also grow.

The peculiar organization of the cytoskeleton in HCL may explain the tendency of these cells to adhere spontaneously in vitro without any artificial "coating" of the substratum. The in vitro adhesive properties of HCL cells may provide an explanation for the peculiar clinical behavior of the disease, which, in its typical form, presents very low numbers of circulating malignant cells but a marked infiltration of both bone marrow (BM) and peripheral lymphoid organs, notably the spleen.[40,41] B-CLL cells relentlessly accumulate in the BM and lymphoid organs and fail in large part to recirculate.[42] These cells, when cultured in vitro, adhere spontaneously to glass coverslips and do show adhesion structures. Our in vitro data may represent the experimental counterpart of the B-CLL sluggish recirculation in vivo.

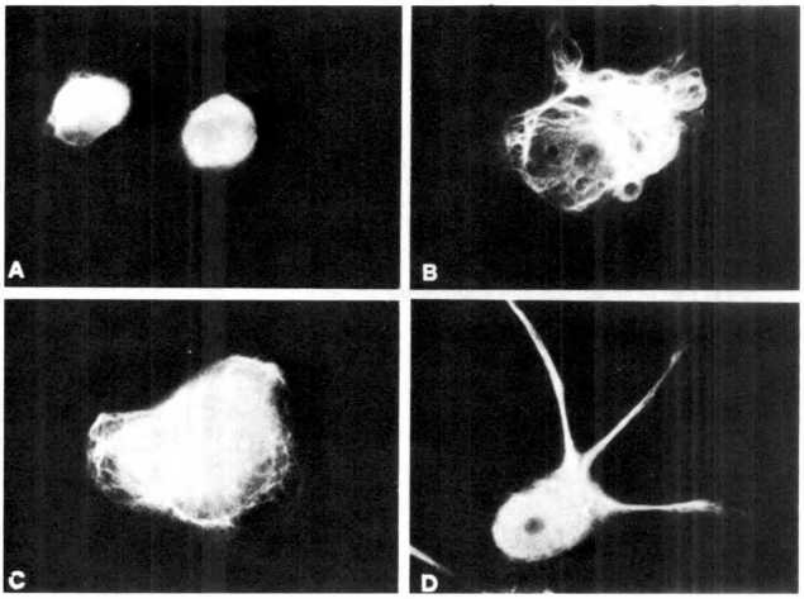

Fig 4. Immunofluorescence localization of vimentin in uninduced (A) and TPA-induced B cells (B), B-CLL (C), and HCL cells (D). Individual vimentin filaments cannot be resolved in normal B cells (A), while they can be observed after induction (B) also in B-CLL cells (C). Vimentin filaments do not extend into thin cellular processes but do so in the long dendritic extensions of TPA-induced HCL cells (D). (Original magnification ×3,500; current magnification ×2,275)

The second observation is that the substratum adhesion of B-CLL cells is not mediated by the conventional focal contacts (also called "adhesion plaques") described in other cell types, eg, in fibroblasts.[43] Rather, adhesion is mediated by dot-shaped close contacts that have been earlier described in a variety of transformed cells[44-46] and recently characterized and called podosomes.[33] Podosomes concentrate the bulk of cellular actin and other cytoskeletal proteins in short protrusions of the ventral membrane identified by electron microscopy.[33] In B-CLL cells, the major portion of cellular F-actin is localized in podosomes, but some is found also submembraneously in peripheral and dorsal ruffles. There is a substantial lack of bundled microfilaments, and therefore, stress fibers are not observed.

The expression of podosomes and the distribution of cytoskeletal proteins appear as a continuum in uninduced B-CLL, TPA-induced B-CLL, and HCL cells. This finding further supports the existence of a close link between B-CLL and HCL. However, some points still have to be clarified. In uninduced B-CLL cells, podosomes are scattered, but after induction, they consistently cluster in a sharply defined area of the ventral membrane sitting beneath the nucleus and sometimes displacing it. The mechanism(s) that governs this event is unknown. Unknown as well is the reason why some cases of TPA-induced B-CLL cells sprout long dendritic processes identical to those shown by HCL cells after TPA. Also, the relationship between podosomes and dendritic processes bringing about a full redistribution of intracellular actin is not yet understood. The mechanics of these events should be looked for in the pleiotropic actions of TPA (for review, see refs 21 and 39) and in the largely unknown cascade of events following the activation of protein kinase C.[15,16]

The third observation is that bona fide podosomes are not observed in normal lymphocytes at rest or after TPA induction. So far, the only nontransformed cells that have been shown to adhere by means of podosomes are osteoclasts[47] and some monocyte-derived cells, including macrophages (ref 48 and P.C. Marchisio, F.G. Giancotti, A. Tefi, A. Zambonin-Zallone, and G. Tarone, manuscript in preparation). However, the number of monocytes in our samples was almost negligible compared with the number of adherent cells. Also, monocytic markers could not be detected in B-CLL cells both uninduced or after TPA treatment. B-CLL cells express monoclonal Igs on their surface or within the cytoplasm and stain with the B cell-specific Mo Abs B1, B4, and RFB4. They fail to react with the monocyte-specific Mo Abs Mo1, Mo2, and UCHM1 and do not exhibit cytoplasmic nonspecific esterase activity. HCL cells have been shown by gene rearrangement studies to belong to the B cell lineage,[49,50] although with some unusual features, such as a strong adhesive tendency (but see earlier) and the ability to phagocyte latex particles.[51] They are also nonspecific esterase negative.

In conclusion, podosomes are adhesion structures that appear associated with B-CLL and (partially with) HCL malignant B lymphoid cells but are absent in their normal counterparts. It is, therefore, important to point out that podosomes have been found also in mammalian and avian cells transformed by Rous sarcoma virus.[33,44-46] In transformed cells, podosomes are peculiar adhesive structures that concentrate tyrosine-phosphorylated proteins,[4] including the specific tyrosine kinase pp60v-src.[45] Thus, it is tempting to suggest that these cytoskeletal structures may represent one hallmark of transformation also in B-CLL cells. The transformation of a normal B lymphocyte into a neoplastic B-CLL cell might be marked by a rearrangement of its membrane-microfilament interactions, leading to the appearance of novel adhesion properties. Whether the expression of cellular oncogenes is responsible for the whole pattern of cytoskeletal changes in these cells is a promising avenue for future investigations.

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Platelet Modulation of Polymorphonuclear Leukocyte Shear Induced Aggregation

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A cone and plate viscometer and Coulter Counter were used to study platelet modulation of polymorphonuclear leukocyte (PMNL) aggregation caused by controlled shear stress. As an index of aggregation, the large-particle percentage (LPP) was calculated. This represents the ratio of aggregated cell count to total cell count. PMNL suspensions in buffer (1.0×10^7 cells per milliliter, final concentration) did not show any aggregate formation at shear stresses below 150 dynes/cm^2 for one minute exposure time (LPP $<$ 3%). However, there was PMNL aggregation in mixed PMNL and platelet-rich plasma suspensions in this shear stress range. Supernatant plasma from sheared platelets initiated PMNL aggregation at moderate shear stress (150 dynes/cm^2 for one minute; LPP, 20.3% ± 2.5%). In contrast, platelet release factors, such as adenosine diphosphate (2 μmol/L) and serotonin (2 μmol/L) did not cause PMNL aggregation (LPP, 2.9% ± 1.2% and 3.3% ± 0.8%, respectively). The use of a cyclo-oxygenase inhibitor (acetylsalicylic acid, 50 μmol/L) did not suppress the aggregation of PMNLs after shear (LPP, 20.1% ± 2.4%). However, preincubation with nordihydroguaiaretic acid (10 μmol/L), an inhibitor of C-5 and C-12 lipoxygenase, and 6,9-deepoxy-6,9-(phenylimino)-6,8-prostaglandin I_1 (U-60257, 10 μmol/L), an inhibitor of C-5 lipoxygenase in human leukocytes, suppressed this aggregation (LPP, 9.1% ± 2.5% and 10.4% ± 3.2%, respectively). Also, the formation of lipoxygenase products (5-HETE, 12-HETE, 15-HETE, and LTB_4) activated by shear stress was documented by reversed phase-high-performance liquid chromatography (RP-HPLC). These data support the possibility of a cooperation between platelets and leukocytes in shear-induced PMNL aggregation that is dependent on C-12 or C-5 lipoxygenase activity, or both.

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POLYMORPHONUCLEAR leukocytes (PMNLs) are a central component of cellular response in inflammatory reactions and of paramount importance to the immune surveillance system. These phagocytic cells contain diverse tissue-destroying and microbicidal enzymes and proteins. Chemotactic factors appear pivotal in this migratory process. Many chemotactic factors have been described, but those derived from the fifth component of complement (eg, C5a and des-Arg-C5a) and synthetic analogues of the putative bacterial chemotactic factor (eg, *N*-formyl peptides) have been most thoroughly characterized.[1-6] These agents, in the nanomolar concentration range, bind and activate the PMNLs by interacting with distinct receptor sites to induce directional migration of the PMNLs. They also stimulate other cellular functions, such as aggregation, adherence, enzyme release, and the production of toxic oxygen metabolites. Thus, in addition to attracting PMNLs, chemotactic factors appear capable of modulating a number of other aspects of the inflammatory reaction. Several chemotactic factor-induced PMNL aggregation studies have been performed, initially stimulated by the observation of neutropenia in extracorporeal hemodialysis and endotoxemia. Neutropenia, pulmonary sequestration, and arterial hypoxemia are all transient phenomena exhibiting a time course that appears to parallel that of PMNL aggregation in vitro.[1-3]

Fluid shearing is known to produce cell interactions and, in whole blood, can lead to platelet–leukocyte aggregation.[7,8] However, no studies have been reported of shear-induced PMNL aggregation under controlled fluid mechanical conditions. Improved designs for artificial organs and prostheses require more detailed knowledge of thromboembolic phenomena produced by mechanical trauma and biomaterial surface exposure. Shear stresses of greater than 150 dynes/cm^2 can be generated during blood flow through hemodialysis devices, artificial heart valves, and blood oxygenators. Unfortunately, the complex and variable shear stress histories that cells experience in cardiopulmonary bypass and other circulatory support systems are hard to quantitate. Therefore, to generate an understanding of the role of leukocytes in embolic phenomena initially requires performing experiments in which the fluid forces are well known. In vitro studies of shear stress and surface interaction effects have normally used simpler flows, including stagnation point flow, flow through tubes, and flow in rotational viscometers. In this study, a rotational cone and plate viscometer was used for the purpose of applying a known uniform shear stress to the entire sample volume.

PMNLs may participate along with platelets in hemostasis and thrombosis.[7,9] In platelets, the cyclo-oxygenase pathway of arachidonic acid metabolism has been implicated in these processes. Platelets also have a pathway involving lipoxygenase action on arachidonic acid, which forms (12S)-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) and, subsequently, the corresponding (12S)-12-hydroxy acid (12-HETE).[10-12] The main pathway of arachidonic acid metabolism in neutrophils involves 5-lipoxygenation, leading to the formation of eicosanoids such as leukotrienes (LTs) and other hydroxy acids.[13-16] The discovery of LTs, a new family of bioactive metabolites formed in a lipoxygenase—type reaction (involving the C-5 of arachidonic acid), and the detection of a C-15 lipoxygenase in leukocytes have indicated the existence of several lipoxygenase enzymes in blood cells. Arachidonic acid does not normally exist in free form but is usually esterified in

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triglycerides and phospholipids. Stimulated phospholipases can release arachidonic acid, which is then enzymatically metabolized by the 5-lipoxygenase pathway to form LTs as well as a host of other bioactive metabolites.[16–18] In addition to differences in positional specificities, the heterogeneity of these enzymes is further emphasized by different reactivities. For instance, it has been shown that, in contrast to platelet suspensions, which can efficiently transform exogenous arachidonic acid into 12-HETE and cyclo-oxygenase products, the addition of the fatty acid to preparations of human PMNLs was followed by only minor transformation of the substrate.[4,13] However, the metabolism of arachidonic acid was strongly enhanced when leukocytes were incubated in the presence of arachidonic acid and divalent cation ionophore A23187, indicating clearly that the human PMNL C-5 lipoxygenase required activation for 5-HETE and LT synthesis. Maclouf et al[10] have shown that platelet-derived 12-HPETE can stimulate this C-5 lipoxygenase. Recent studies suggest the involvement of LTs in hypersensitivity reactions and inflammation and point out the importance of a better understanding of the biochemical process involved in the control of the biosynthesis of these bioactive substances.[13,19–23]

To begin to understand the biochemical control mechanisms of shear-induced PMNL aggregation, we have used inhibitors of cyclo-oxygenase or lipoxygenase (acetylsalicylic acid [ASA], nordihydroguaiaretic acid [NDGA], U-60257), synthetic chemotactic tripeptide formyl-methionyl-leucyl-phenylalanine (FMLP), and calcium ionophore A23187. The effect of these factors in PMNL aggregation in the presence or absence of platelets and shear-activated platelet-released products suggests the occurrence of significant biochemical interactions between platelets and leukocytes in mechanically induced activation processes.

MATERIALS AND METHODS

Preparation of human PMNLs. Venous blood from normal healthy donors was drawn by venipuncture into heparin (10 U/mL, final concentration) in conical plastic tubes. PMNLs were purified using standard techniques of dextran sedimentation, centrifugation on Ficoll-Hypaque, and hypotonic lysis of erythrocytes. Final cell suspensions contained a minimum of 95% PMNLs. Viability of the PMNLs was always greater than 95% as determined by trypan blue exclusion. PMNLs were labeled with 5.5 µmol/L of 1-^{14}C arachidonate added during the first wash.

Preparation of platelet-rich plasma (PRP). Blood was obtained by venipuncture of healthy volunteers. Ten milliliters of blood was quickly and gently mixed with 1.1 mL of heparin (100 U/mL) in siliconized glass tubes. The heparinized blood was centrifuged for ten minutes at 150 g. The supernatant PRP was drawn off with a Pasteur pipette and transferred to a clean siliconized glass or plastic test tube. The remaining fractions were centrifuged for five minutes at 150 g to retrieve more PRP. Finally, the red blood cell fraction was centrifuged for ten minutes at 1,000 g. The resultant supernatant was platelet-poor plasma (PPP) and was transferred to a separate test tube. Platelet counts in all PRP samples were adjusted to 3×10^8/mL, final concentration, by dilution with PPP.

Reagents and buffers. 1-^{14}C Arachidonate (52.0 mCi/mmol, specific activity), ^{3}H 6-keto PGF$_{1\alpha}$, ^{3}H thromboxane B$_2$, ^{3}H PGF$_{2\alpha}$, ^{3}H PGE$_1$, ^{3}H PGE$_2$, and ^{3}H PGD$_2$ were all purchased from New England Nuclear (Boston). 5-HETE radioimmunoassay kit was purchased from Seragen (Boston). A23187, adenosine diphosphate (ADP), ASA, NDGA, FMLP, cytochalasin B, and colchicine were purchased from Sigma Chemical Co (St Louis). A23187, NDGA, FMLP, and colchicine were dissolved in dimethylsulfoxide (DMSO). Cytochalasin B and ASA were prepared in ethanol. The 6,9-deepoxy-6,9-phenylimino-6,8-prostaglandin I$_1$ (U-60257) was kindly provided by Dr M.K. Bach of the Upjohn Company and prepared in tris(hydroxymethyl)aminomethane (THAM). The small amount of ethanol and DMSO (final concentration of 0.1%) used as vehicles did not alter cell viability. The buffer was Dulbecco's phosphate-buffered saline (PBS) containing (mmol/L): NaCl (137), KCl (2.7), $Na_2HPO_4 \cdot 7H_2O$ (8.1), KH_2PO_4 (1.9), $CaCl_2$ (0.9), and $MgCl_2 \cdot 6H_2O$ (0.5).

Aggregation assay. The isolated PMNLs were suspended (1×10^7 cells per milliliter) in PBS and incubated according to the various procedures described under Results. After one minute of shearing at 150 dynes/cm^2, 20-µL samples were taken from the suspensions. The samples were immediately diluted in 10 mL of Isoton solution (Coulter Electronics, Hialeah, Fla) and analyzed in the Coulter Counter system. The Coulter Counter was a Model ZBI and was connected to Coulter Channelyzer and microcomputer. For each sample, the particle concentration was enumerated with a lower channel of 150 µm^3 (called the total or "T" particle concentration) and with a lower channel of 540 µm^3 (called the aggregated or large "A" particle concentration).[1,2] These names are given because with the lower channel of 150 µm^3, all PMNLs in a sample were counted, whereas with a lower channel of 540 µm^3, only particles larger than 1.8 times the size of an average unaggregated PMNL were enumerated. Results for a given sample of the PMNL suspension were reported as the large-particle percentage ($LPP = A/T \times 100$) or the maximum large-particle percentage. Sheared samples (and others incubated with various chemicals) were analyzed at ten-minute intervals to follow the aggregation process. The maximum large-particle percentage was the peak value of LPP obtained over the 60-minute aggregation interval.

Shear stress application. Leukocyte suspensions and PRP were subjected to shear stress in a specially modified Ferranti-Shirley Viscometer.[8] The platens used gave a cone and plate geometry. Thus the entire sample experienced the same constant shear stress. After shearing PRP for one minute at 150 dynes/cm^2, the sample was removed and rapidly centrifuged to produce a platelet-free supernatant. This supernatant plasma was added to PMNL suspensions within 30 seconds.

High-performance liquid chromatography (HPLC) analysis of icosanoids. In preparation for reversed phase-HPLC (RP-HPLC), cell suspensions were extracted by using Sep-PAK C18 cartridges

Table 1. The Mixing Ratio and Flow Rate Changes in the HPLC

Time (min)	A* (%)	B† (%)	C‡ (%)	Flow Rate (mL/min)
0.0	72	0	28	1.5
3.0	65	0	35	1.0
11.0	65	0	35	1.0
16.0	50	10	40	1.3
39.5	46	14	40	1.3
44.5	45	15	40	1.5
47.0	42	15	43	1.0
55.0	0	0	100	2.0
65.0	0	0	100	2.0
70.0	72	0	28	1.0

*Distilled H_2O (0.1% HOAC), pH 3.2.

†CH_3OH.

‡CH_3CN.

and 90% methanol eluates were evaporated under nitrogen. Residues were dissolved in 100 μL of methanol, and 10 μL was injected onto the HPLC column. RP-HPLC was carried out on a 4-mm ID μ-PAK C18 (5 μm) column (Varian Assoc Instrument, Palo Alto, Calif) with water–methanol–acetronitrile as the eluting solvent. Table 1 gives the mixing ratio and flow rate changes as functions of elution time. Fractions were collected, and the radiolabel content of each was determined in Hydrofluor by liquid scintillation counting. Identification of the peaks was done by comparison with standards. Under these conditions, stable cyclo-oxygenase products 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), thromboxane B_2 (TXB_2), $PGF_{2\alpha}$, PGE_1, PGE_2, and PGD_2 were eluted at four to ten minutes; LTB_4 at 20 to 22 minutes; mono-HETEs at 40 to 52 minutes, and unreacted arachidonate at 55 to 60 minutes.

RESULTS

Chemotactic factor and ionophore-induced PMNL aggregation. FMLP, as previously described by others,[1,2] aggregated human PMNLs. This effect was reflected in an increase in the percentage of large particles formed after adding 1 μmol/L FMLP to the suspension of PMNLs (Fig 1). Cytochalasin B, by itself, did not aggregate the cells but did significantly enhance FMLP-induced aggregation (Fig 1). The calcium ionophore A23187 (10 μmol/L) aggregated PMNLs (Fig 1), and the time course of aggregation was similar to that of shear-induced PMNL aggregation in the presence of supernatant from sheared PRP (see below). The kinetics of FMLP-induced aggregation reached nearly a maximum LPP within two to three minutes. However, the time course of ionophore A23187- and shear-induced aggregation was much slower, and a maximum value was not attained for over 30 minutes.

Shear-induced PMNL aggregation. PMNL suspensions in PBS (1×10^7 cells per milliliter, final concentration) did not show any aggregate formation at shear stresses below 150 dynes/cm^2 for one minute (Figs 2 and 3). However, when PMNLs were incubated in the presence of PRP, a strong stimulation of PMNL aggregation occurred in this shear stress range. PMNLs incubated with supernatant plasma from sheared platelets (1:1 by volume) would initiate PMNL aggregation after exposure to moderate shear stress (150 dynes/cm^2 for one minute, Figs 2 and 3). The effect of plasma protein on the shear-induced PMNL aggregation was examined by using the PMNL suspensions mixed with platelet-free plasma (PFP) (1:1 by volume, Figs 2 and 3). Platelet release factors, 2 μmol/L ADP or 2 μmol/L serotonin, did not induce PMNL aggregation at this shear stress (Fig 3).

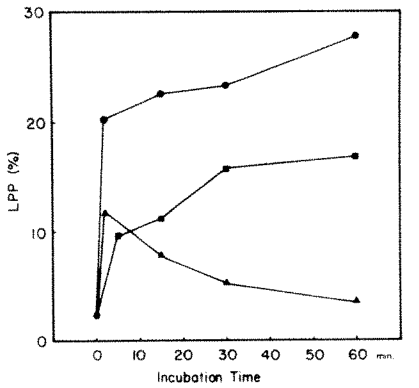

Fig 1. The kinetics of the chemotactic factor and ionophore-induced PMNL aggregation. PMNLs (1×10^7 cells per milliliter) were incubated with 1 μmol/L FMLP (▲) and 10 μmol/L A23187 (■) for the specific time periods. PMNLs were preincubated with cytochalasin B (5 μg/mL) for five minutes and then incubated with 1 μmol/L FMLP (●).

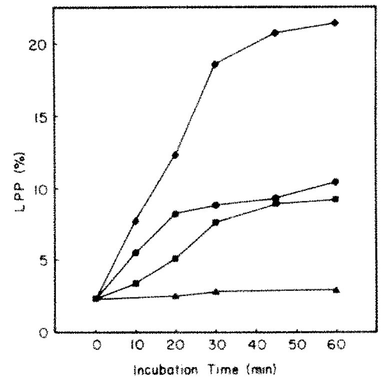

Fig 2. The kinetics of the shear-induced PMNL aggregation. PMNLs (▲), PMNLs mixed with platelet-free plasma (PFP) (●), PMNLs mixed with supernatant plasma of sheared platelets (2.5 to 4.0×10^8 cells per milliliter) (♦), and the last sample preincubated with 10 μmol/L NDGA (■) were sheared at 150 dynes/cm^2 for one minute. After shearing, samples were analyzed at ten-minute intervals for one hour to follow the aggregation process.

To define the nature of the factor(s) responsible for the effect of sheared platelet supernatants on leukocyte aggregation activity, experiments with inhibitors were performed. The inhibition of the cyclo-oxygenase pathway by 50 μmol/L ASA did not suppress the aggregation of PMNLs after shear (Fig 4). However, preincubation with NDGA (10 μmol/L), an inhibitor of C-5 and C-12 lipoxygenase, and U-60257 (10 μmol/L), an inhibitor of C-5 lipoxygenase in human leukocyte,[24,25] suppressed the aggregation of PMNLs (Figs 2 and

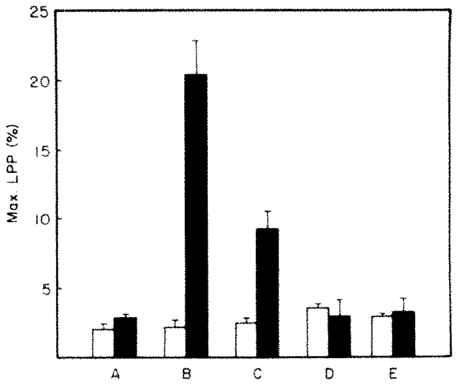

Fig 3. Effect of platelet release products on shear-induced PMNL aggregation. (A) PMNLs in buffer alone, (B) PMNLs mixed with supernatant plasma of sheared platelets, (C) PMNLs mixed with PFP, (D) PMNLs incubated with 2 μmol/L ADP, and (E) PMNLs incubated with 2 μmol/L serotonin. Sheared samples (150 dynes/cm^2 for one minute, closed bar) were compared with control samples (unsheared, open bar). Data represent the mean ± SEM for three to nine separate experiments and are the maximum LPP over a one-hour interval after shear exposure.

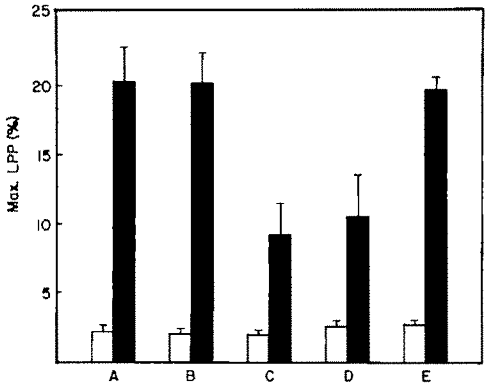

Fig 4. Effect of cyclo-oxygenase and lipoxygenase-inhibitors on PMNL shear-induced aggregation. PMNLs were mixed with supernatant plasma of sheared platelets and incubated with no inhibitors (A), ASA (50 μmol/L) (B), NDGA (10 μmol/L) (C), U-60257 (10 μmol/L) (D). In (E), PRP was incubated with imipramine (1 μmol/L) and sheared, and subsequently, the PMNLs were mixed with this supernatant. Sheared samples (150 dynes/cm² for one minute, closed bar) were compared with control samples (open bar). Data represent the mean ± SEM for three to nine separate experiments.

4). Supernatant of sheared PRP, which had been preincubated with NDGA (10 μmol/L), also suppressed subsequent shear-induced PMNL aggregation (data not shown).

PRP incubated with imipramine (1 μmol/L), an inhibitor of serotonin uptake, for 15 minutes was sheared and centrifuged. In this case, serotonin that was released from sheared PRP was in the supernatant. PMNLs incubated with this supernatant did not show any change in the shear-induced PMNL aggregation (Fig 4). These data all suggested that serotonin had no effect in the shear-induced PMNL aggregation. In addition, incubation of PMNL with ADP (2 μmol/L) did not induce aggregation, with or without shear stress. Fig 5 shows the effect of shear stress magnitude on PMNL aggregation for three different samples.

Effect of platelet-PMNL ratios. When PMNL and PRP (1:1 by volume) samples were sheared at 150 dynes/cm² for one minute, there were differences in the aggregation profiles dependent on the ratio of platelets to PMNLs (Fig 6). When the ratio was approximately 30, the aggregation response of PMNL was maximum. At this ratio, the total volume of platelets was nearly equivalent to that of PMNLs. Also, this ratio is nearly the same as that found physiologically.

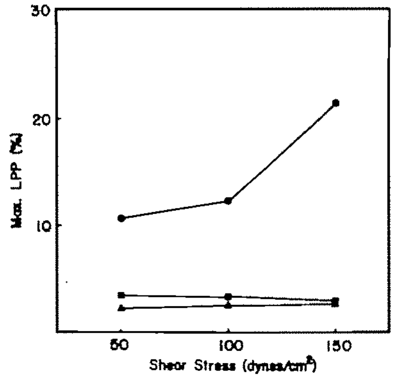

Fig 5. Effect of shear stress magnitude on the maximum LPP. Three shear stresses (50, 100, and 150 dynes/cm²) were used to shear PMNLs in buffer (▲), PMNLs incubated with 2 μmol/L ADP (■), and PMNLs mixed with supernatant plasma of sheared platelets (●).

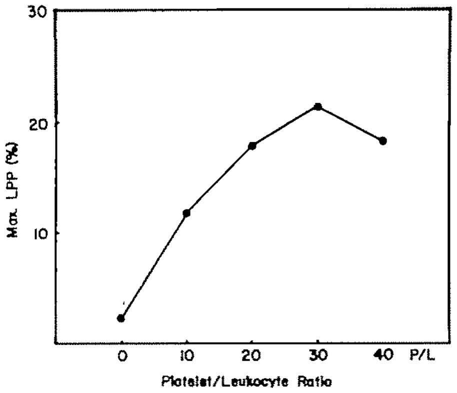

Fig 6. Effect of platelet concentration on shear-induced PMNL aggregation. Different platelet concentrations in PRP were sheared and centrifuged. This supernatant plasma was added to PMNL suspensions (1 × 10⁷ cells per milliliter) and, subsequently, sheared at 150 dynes/cm² for one minute.

HPLC experiments. Studies of platelet or PMNL arachidonate metabolites, identified by HPLC, were used to examine the details of arachidonic acid metabolism under shear stress. Results of a group of experiments analyzed by RP-HPLC are shown in Figs 7 through 10.

PMNL suspensions mixed with 5.5 μmol/L 1-^{14}C arachidonic acid and supernatant of sheared PRP generated a significantly increased quantity of LTB$_4$ after shearing, which appeared as a distinct peak (retention time, 21 minutes), as shown in Fig 7. When PMNL suspensions were preincubated with 10 μmol/L NDGA, there was no significant increase of LTB$_4$ formation after shearing (Fig 8). Only 12-HETE formation was increased when PRP and 5.5

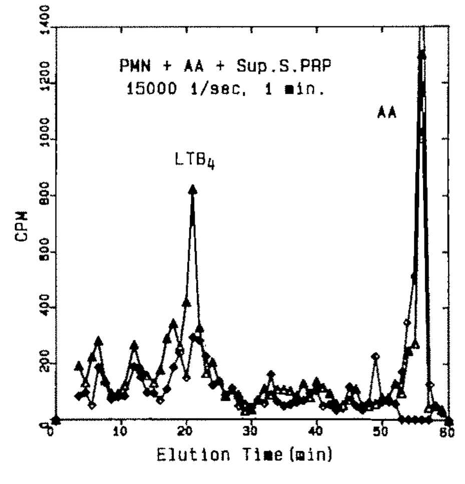

Fig 7. RP-HPLC chromatogram of the metabolites of arachidonic acid formed in human PMNLs incubated with 5.5 μmol/L 1-^{14}C arachidonic acid in the presence of supernatant of sheared PRP. Control (◇) and after shearing at 150 dynes/cm² for one minute (△).

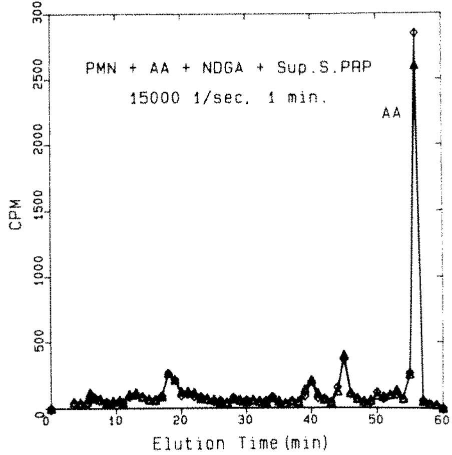

Fig 8. RP-HPLC chromatogram of the metabolites of arachidonic acid formed in human PMNLs preincubated with 10 μmol/L NDGA and with 5.5 μmol/L 1-^{14}C arachidonic acid in the presence of supernatant of sheared PRP. Control (◇) and after shearing at 150 dynes/cm^2 for one minute (△).

μmol/L 1-^{14}C arachidonic acid were exposed to shear stress (Fig 9). That this metabolite was 12-HETE, not 5-HETE, was confirmed by using thin-layer chromatography (data not shown). Little cyclo-oxygenase products were detected after shear stress stimulation of platelets. The amount of TXB_2 formation after shear stress stimulation of platelets measured by radioimmunoassay was ≤10 ng/10^8 platelets. The counts per minute (cpm) range corresponding to this concentration was nearly the same as the background cpm.

The amount of shear-induced 5-lipoxygenase products (LTB_4, 5-HETE) in sheared PMNL suspensions alone was much less than when supernatant of sheared PRP was present (data not shown). In fact, LTB_4 production under these conditions was negligible. Shear-induced 5-HETE production in the PMNL suspensions has also been quantitated using radioimmunoassay with labeled antibody purchased from Seragen. The formation of 5-HETE increased proportionally with the shear stress increase (data not shown). PMNLs and 5.5 μmol/L 1-^{14}C arachidonic acid were incubated with 10 μmol/L ionophore A23187 in the presence of sheared PRP supernatant. In this case, production of LTB_4 by PMNLs after shearing increased even more dramatically. It is noteworthy that 5-HETE production was increased and 15-HETE formation was decreased (Fig 10).

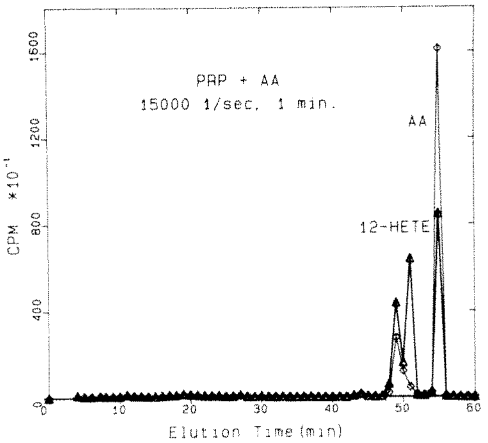

Fig 9. RP-HPLC chromatogram of the metabolites of arachidonic acid formed in PRP incubated with 5.5 μmol/L 1-^{14}C arachidonic acid. Control (◇) and after shearing at 150 dynes/cm^2 for one minute (△).

DISCUSSION

In this paper, we have shown that a substance(s) derived from platelet activation in a shear field may activate the C-5 lipoxygenase of human blood PMNLs in vitro. However, there is an activation (shearing) requirement for PMNL aggregation even though the cells have been incubated with supernatant plasma of sheared PRP (Fig 3B). PMNLs incubated with plasma alone show some shear-induced aggregation (Fig 3C). This may be due to the plasma protein binding and, to a lesser extent, viscosity changes. The difference between B and C in Fig 3 indicates that a substance(s) derived from shear activated platelets plays an active role in this PMNL aggregation.

Sheared platelets release ADP and serotonin, which are potent platelet-aggregating agents. However, 2 μmol/L ADP and 2 μmol/L serotonin do not cause PMNL aggregation (Fig 3D and E, respectively). Redl et al[26] recently demonstrated that platelets augment granulocyte aggregation by a serotonin and thromboxane A_2-independent mechanism, and it was not necessary for platelets to be intact for them to augment granulocyte aggregation. Indeed, platelet lysates and the supernatant fluids therefrom proved equally efficacious. NDGA (10 μmol/L) and U-60257 (10 μmol/L) totally suppressed the shear-induced PMNL aggregation to that level seen in a mixture of PMNL and PFP (Fig 4C and D, respectively). It is hypothesized that the platelet-derived PMNL activating substance may be 12-HPETE. Shear-induced aggregation of platelets produces the release of arachidonic acid from cellular phospholipids and the subsequent metabolism of the arachidonic acid by cyclo-oxygenase and lipoxygenase systems. It is interesting that in

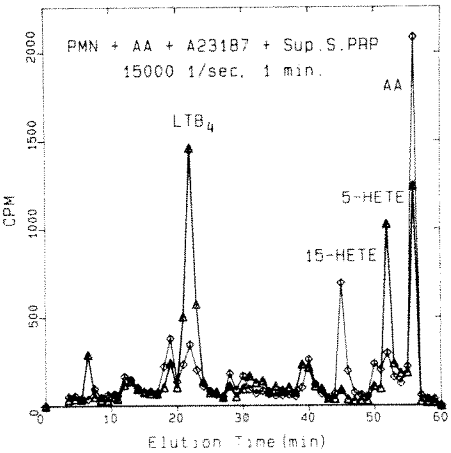

Fig 10. RP-HPLC chromatogram of the metabolites of arachidonic acid formed in human PMNLs incubated with the ionophore A23187 (10 μmol/L) and 5.5 μmol/L 1-^{14}C arachidonic acid in the presence of supernatant of sheared PRP. Control (◇) and after shearing at 150 dynes/cm^2 for one minute (△).

shear activation, a substantial portion of the free arachidonic acid is transformed by a lipoxygenase to 12-HPETE and, subsequently, 12-HETE (Fig 9). This 12-HPETE may activate the C-5 lipoxygenase in PMNLs and therefore lead to increased release of C-5 lipoxygenase products, such as 5-HETE and LTs. Figure 11 shows a hypothetical scheme of the platelet–PMNL interactions that may lead to increased release of leukocyte C-5 lipoxygenase products.

It has been shown that the C-15 lipoxygenase also interacts with the C-5 lipoxygenase to produce a (5S, 15S)-5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid (5S, 15S diHETE).[10] The formation of the 5S, 12S diHETE and of the 5S, 15S diHETE reflects the interactions between lipoxygenase enzymes in different blood cells. Marcus et al[27] have shown a new metabolite of arachiconid acid, formed during interaction between thrombin- or collagen-stimulated platelets and unstimulated neutrophils. That was confirmed to be 12S, 20-dihydroxyeicosatetraenoic acid (12S, 20 diHETE). Their experiments showed that platelet stimuli known to occur in vivo might initiate metabolic interactions between different cell types by way of the arachidonic acid pathway. Leukotriene B_4 (LTB_4) is a potent chemotactic factor and induces PMNL aggregation. The stimulation of LT synthesis in human blood leukocytes by 12-HPETE has been observed.[10] Plasma protein binding in our system may stabilize 12-HPETE, prolonging its activity over that seen in buffers alone. LT biosynthesis requires enzymes that are distributed among different subcellular compartments, and it appears that their biosynthesis involves movement of intermediates between these compartments.[28] In the case of LTB_4 formation, the action of a soluble cytosolic hydrolase has been proposed to be the rate-limiting step.[17] Consequently, intermediate substrates such as 5-HPETE or LTA_4 must accumulate, decay by way of alternate enzymatic and nonenzymatic processes, or be released from cells, such as PMNLs. In the latter instance, a plasma enzyme capable of metabolizing extracellular LTA_4 to LTB_4 seems reasonable. In fact, recent studies suggest that an extracellular enzymatic transformation of LTA_4 into the potent chemotaxin LTB_4 may be involved.[29,30] Plasma enzymatic activity responsible for LTB_4 production may play a general role in modulating the inflammatory response.

The 5-lipoxygenase is present in PMNL in an inactive form and must be activated to catalyze the dioxygenation of arachidonic acid. Evidence that the lipoxygenase pathway might be involved in PMNL activation comes from the studies showing that rabbit exudate PMNLs are stimulated directly by exogenous arachidonic acid to release HETEs and LTB_4[4,14] and to aggregate.[31] In a recent study, it was shown that human PMNLs, isolated from normal individuals, are stimulated when given a combination of arachidonic acid and ethanol, which apparently activates the 5-lipoxygenase pathway for PMNLs. However, the addition of arachidonic acid alone to human blood PMNL suspensions does not induce significant synthesis of 5-lipoxygenase products.[4]

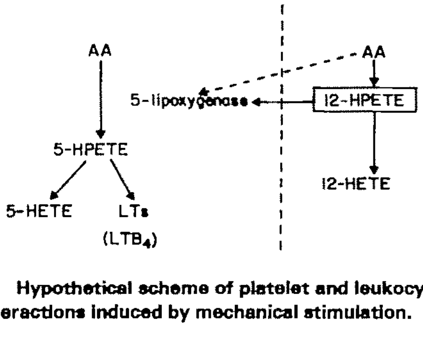

Fig 11. Hypothetical scheme of platelet and leukocyte lipoxygenase interactions induced by mechanical stimulation.

The divalent cation ionophore A23187 is known to be a potent activator of the synthesis of LTs. Its action probably involves the stimulation of Ca^{2+} flux and the subsequent activation of the Ca^{2+}-dependent 5-lipoxygenase and phospholipases. It thus appears that the release of arachidonic acid (after phospholipase stimulation) and the activation of the 5-lipoxygenase are prerequisites for the synthesis of 5-lipoxygenase products in human blood PMNLs. One possible mode of action of stress-induced mechanical activation is a membrane mechanical stretching induced increased calcium flux, leading to increased intracellular enzymatic activity. Also, Volpi et al[32] have shown that the addition of arachidonic acid to neutrophils will cause a rapid and significant increase in the permeability of the plasma membrane to calcium. This hypothesis is supported by the similarity of A23187-induced (Fig 1) and shear-induced (Fig 2) PMNL aggregation time course curves.

Quantitative confirmation of lipoxygenase involvement in the shear-induced aggregation process was obtained using RP-HPLC. The formation of LTB_4 was greatly increased after shearing PMNL in the presence of supernatant plasma from sheared platelets (Figs 7 and 10). When the leukocytes were stimulated both by ionophore A23187 and shearing, LTB_4 and 5-HETE production increased dramatically. However, preincubation of 10 μmol/L NDGA with PMNL suspensions totally suppressed the increment of LTB_4 formation after shearing (Fig 8). This again supports the hypothesis that these 5-lipoxygenase products induce PMNL aggregation.

Small amounts of diHETEs were detectable when PMNL suspensions were sheared in PFP or in buffers. Sheared platelets exhibited greatly increased synthesis of 12-HETE (Fig 9) with surprisingly little thromboxane B_2 production. This indicates that shear stress stimulation of platelets may produce quite different arachidonic acid metabolite than that seen with direct chemical stimuli, such as thrombin or the ionophore A23187. This platelet-derived 12-HETE (12-HPETE) may be the mediator that stimulates the 5-lipoxygenase activity in human PMNLs during stress-induced aggregation. The fact that preincubation of PRP with a 12-lipoxygenase inhibitor (NDGA, 10 μmol/L) before shearing blocked the effect of sheared-platelet supernatant on PMNL aggregation strengthens our hypothesis.

The platelet-PMNL ratio appears to be important (Fig 6). When the ratio is approximately the same as found under physiological conditions, the aggregation response of the PMNL is maximum. It is possible that, in addition to the platelet-PMNL ratio, the cell concentration in the incubation medium is an important parameter. This has been observed in studies examining the possibility of platelet-derived endoperoxides serving as substrates for endothelial cell prostacyclin synthase.[33]

In summary, our studies suggest important roles for the platelet and PMNL C-12 and C-5 lipoxygenases in shear-induced PMNL aggregation. These data support the concept of platelet–PMNL interactions in shear-induced PMNL aggregation and add new data concerning the potential role of release products from one cell type in modulating the biological and mechanical response of another blood cell.

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CONCISE REPORT

Analysis of T-Cell Receptor Beta Chain (T_β) Gene Rearrangements Demonstrates the Monoclonal Nature of T-Cell Chronic Lymphoproliferative Disorders

By Robin Foa, Pier-Giuseppe Pelicci, Nicola Migone, Francesco Lauria, Giovanni Pizzolo, Frances Flug, Daniel M. Knowles II, and Riccardo Dalla-Favera

We investigated the rearrangement patterns of the gene coding for the beta chain of the T cell receptor (T_β) in 11 patients with T-cell derived chronic lymphoproliferative disorders, including T-cell prolymphocytic leukemia (T-PLL) and T-cell chronic lymphocytic leukemia (T-CLL). We found that all five cases of T-PLL, and five of six cases of T-CLL, displayed T_β-gene rearrangements, clearly establishing their monoclonal nature. Clonality could not be determined in one case of T-CLL where the T_β gene was found unrearranged. Our results demonstrate that the majority of cases of both clinically aggressive T-PLL and clinically indolent T-CLL are monoclonal. These results suggest that the analysis of T_β gene rearrangements represents a valid tool for the differential diagnosis and clinical monitoring of T-cell derived chronic lymphoproliferative diseases.

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T-CELL chronic lymphoproliferative disorders are well recognized clinicopathologic diseases, which include T-cell prolymphocytic leukemia (T-PLL) and T-cell chronic lymphocytic leukemia (T-CLL). T-PLL is usually characterized by clinical features suggesting a malignant process, ie, a high WBC count, marked splenomegaly, an aggressive clinical course and a limited therapeutic response.[1] T-CLL, including large granular lymphocyte (LGL) proliferations (T_γ-lymphoproliferative disease, T_γ-LPD),[2] are generally characterized by a relatively low WBC count, an indolent, nonprogressive clinical course and prolonged survival.[2,3] The benign clinical course of many cases of T-CLL has led some authors[2,4] to question their neoplastic nature and to recommend the term chronic T-cell lymphocytosis[4] unless disease progression is demonstrable. Difficulty in determining the neoplastic or the reactive nature of these cases has stemmed from the lack of an adequate marker of clonality for T-cell populations.

Recently, DNA sequences coding for the beta chain of the T-cell receptor (T_β) have been isolated. It has been shown that the T_β locus undergoes structural rearrangements that precede and are necessary for T_β gene expression in T-cells,[5-8] analogous to immunoglobulin gene rearrangements in B-cells.[9,10] We and others have demonstrated that the patterns of T_β gene rearrangement, identified by Southern blot hybridization analysis using T_β gene probes,[11-13] can be used to identify the mono- or polyclonality of a given T-cell population.[12] We used this immunogenotypic approach, in the studies described here, to demonstrate that the vast majority of T-cell chronic lymphoproliferative disorders, including clinically aggressive T-PLL and clinically indolent T-CLL, are monoclonal T-cell proliferations.

PATIENTS AND METHODS

Patients and diagnosis. We studied six cases of T-CLL and five cases of T-PLL. Diagnoses were established by conventional clinical, morphological, and phenotypic criteria. All six patients with T-CLL exhibited a benign clinical course and a moderate lymphocytosis. The cells in five patients (Table 1, cases 2 through 6) displayed typical LGL morphology, ie, abundant azurophilic granular rich cytoplasm, and absent nucleoli. The cells in one patient (Table 1, case 1) were small, mature appearing lymphocytes, without nucleoli and without LGL morphology. All five patients with T-PLL exhibited an aggressive clinical course and a lymphocytosis of intermediate sized cells with moderately basophilic cytoplasm, vesicular nuclei, and prominent nucleoli.

Immunophenotypic analysis. Sheep erythrocyte (E) rosette formation was assayed with Vibrio Cholera neuraminidase-treated sheep erythrocytes. T-cell associated antigen expression was determined by indirect immunofluorescence using murine monoclonal antibodies OKT3, OKT4, OKT6, OKT8, OKT11, and Leu7.[14]

Cytotoxicity assays. NK activity was measured using K562 leukemia cells as target cells, at effector to target cell (E:T) ratios ranging from 25:1 to 100:1, in a four hour ^{51}Cr release assay. For ADCC, ^{51}Cr-labelled TLX9 lymphoma cells, sensitized with rabbit antibody, were used as targets in a four hour test.

Gene rearrangement studies. DNA purification was performed by cell lysis, proteinase K digestion, extraction with phenol/chloroform and precipitation with ethanol.[15] Fifteen micrograms of DNA extracted from each sample were digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, and transferred to a nitrocellulose filter as described.[15] The probe used in this study is represented by a 440 base pair (bp) *Hin*cII fragment representative of the Constant region of the T_β gene ($T_\beta C$). The original clone and the generation of the probes have been described.[6,12]

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Table 1. Diagnoses, Phenotypic Markers, Relevant Clinical Features, and Summary of T_β Gene Studies

			Phenotypic Markers†						Cytotoxicity†		WBC‡		Enlargement of		
Case No.	Diagnosis	T_βGene*	E/T11	T3	T4	T6	T8	Leu7	NK	ADCC	Total	Lymphocytes	Spleen	Liver	Lymph Nodes
1	T-CLL	R	+/NT	+	+	−	+	NT	NT	NT	17.2	75	−	−	−
2	T-CLL	R	+/NT	+	+	−	+	+	−	NT	8.2	65	−	−	−
3	T-CLL	R	−/+	+	−	−	+	+	−	NT	7.4	62	−	−	+
4	T-CLL	R	+/+	+	−	NT	+	+	−	+	25.1	70	+	+	+
5	T-CLL	R	+/+	+	−	NT	+	+	−	+	9.1	73	−	−	+
6	T-CLL	G	+/+	−	−	−	−	±	+	NT	15.2	78	−	−	−
7	T-PLL	R	+/+	+	+	−	−	−	−	NT	59	84	−	−	−
8	T-PLL	R	+/+	−	+	−	−	−	−	NT	79	92	+	−	−
9	T-PLL	R	+/NT	+	+	−	−	−	−	NT	120	90	+	−	−
10	T-PLL	R	+/+	+	+	−	+	−	−	NT	259	99	+	+	+
11	T-PLL	R	+/NT	+	+	−	+	−	−	NT	49	77	+	−	−

*R, rearranged; G, germ-line

†NT, not tested.

‡WBC, white blood cell count expressed as WBC number × $10^3/\mu L$

RESULTS

In general, these 11 cases displayed the typical clinical and phenotypic characteristics of T-CLL or T-PLL (Table 1). In some cases, the cells displayed a relatively infrequent phenotype, the simultaneous expression of T4 and T8. This phenotype is exceptional in T-CLL[2] and infrequent in T-PLL.[16]

We previously demonstrated that the analysis of T_β gene rearrangements by Southern blot hybridization using T_β probes, allows the definition of the lineage, ie, T-cell, and the mono- or polyclonal nature of a given lymphoid cell population.[12] Figure 1 (lanes A) illustrates the pattern of hybridization bands obtained when human DNA from a non-T-cell population is digested with *Eco*RI or *Bam*HI and hybridized to the T_βC probe. The same analysis, performed on DNA obtained from a polyclonal T-cell population, represented by either normal thymocytes or peripheral blood T-lymphocytes, shows the biallelic deletion of the 12.0 kb band upon digestion with *Eco*R1 and a conserved germline pattern upon digestion with *Bam*HI (Fig 1, lanes B). In monoclonal T-cell populations, the restriction pattern of the gene is characterized by the same *Eco*RI band deletion and in addition, by the presence of one or more rearranged band(s), which can be detected by digestion with at least one restriction enzyme (see ref. 12 and below).

We analyzed the six cases of T-CLL and five cases of T-PLL based upon this scheme. Southern blot analysis demonstrated the presence of T_β gene rearrangements in all five cases of T-PLL and in five of six cases of T-CLL (Table 1). All of the cases but one displayed the disappearance of the 12.0 kb *Eco*RI band and the appearance of new hybridization bands upon DNA digestion with *Eco*RI and/or *Bam*HI (or *Hind*III, not shown) restriction enzymes. Three cases displaying T_β gene rearrangements are illustrated in Fig 1. Cases 3 and 11 showed the appearance of rearranged bands upon DNA digestion with both *Eco*RI and *Bam*HI. In Case 3, the absence of the *Eco*RI and *Bam*HI germline fragments together with the appearance of a single rearranged band, suggests the deletion of one allele of the T_β gene and the rearrangement of the other. A rearranged band in Case 10 is apparent only upon DNA digestion with *Bam*HI restriction enzyme. The single case without rearranged bands also showed conservation of the *Eco*RI 12.0 kb band (see 6 in Fig 1), indicating that the T_β gene is unrearranged in these cells. This finding was confirmed by digestion of the same DNA with *Bam*HI (Fig 1, Lane 6) and *Hind*III (not shown) restriction enzymes. Analysis of the immunoglobulin heavy chain locus showed a germline configuration in all 11

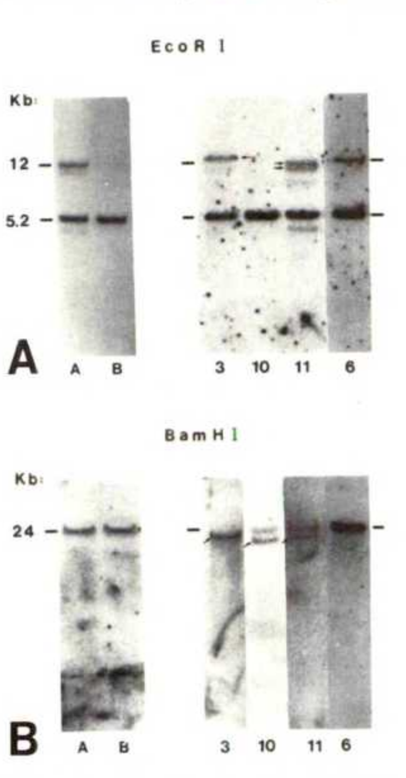

Fig 1. Autoradiographs showing representative results of analysis of T_β gene rearrangements in chronic T-cell derived lymphoproliferative disorders. (A) DNAs from normal human fibroblasts, (B) normal human peripheral blood T-lymphocytes, and from pathologic samples (Cases 3, 6, 10, 11) (see Table 1) were digested with *Eco*RI and *Bam*HI as indicated and hybridized to T_βC probe. Dashes indicate germline bands, arrows indicate rearranged bands.

cases (data not shown). Thus, our data indicate that ten of the 11 T-cell chronic lymphoproliferative disorders are monoclonal T-cell proliferations, since the cells display clonal T_β gene rearrangements. Clonality could not be determined by this approach in one case of T-CLL, since the T_β gene was found to be unrearranged.

DISCUSSION

This study sought to determine the mono- or the polyclonality of T-cell chronic lymphoproliferative disorders, including T-PLL and T-CLL. In contrast with most other neoplasms, the monoclonality of some of these cases, particularly T-CLL, cannot be assumed or easily established in view of: (1) their nonaggressive, clinical behavior; (2) the lack of cytogenetic markers to confirm both their clonality and, by analogy with other neoplasms, their malignancy; and (3) the lack of clearly identifiable morphologic or immunophenotypic features to distinguish them from their normal, benign counterparts.

The studies presented here demonstrate that the vast majority of T-cell chronic lymphoproliferative disorders are monoclonal. The monoclonal and probable malignant nature of T-PLL is strongly suggested by its typical aggressive clinical course. However, the monoclonal nature of T-CLL was also established in five of six cases. The latter cases display a nonaggressive clinical course and immunophenotypic features, which pose reasonable doubts in terms of the differential diagnosis between a benign chronic lymphocytosis and a true lymphoid malignancy. For instance, Case 2 displayed a particularly benign clinical course during three years of follow-up. We also demonstrated that some chronic lymphoid proliferations which display a mixed T4/T8 phenotype are monoclonal. These cases may be more frequent than previously reported,[16] and may have often been inappropriately classified as chronic T-cell lymphocytoses by immunophenotypic analysis.

One case of T-CLL exhibited an unrearranged T_β gene configuration. The cells from this case exhibited LGL morphology and NK function, although their T3- T4- T8- phenotype (Table 1) differed from the other cases of T-CLL displaying LGL features. These observations suggest that LGL-derived T-CLLs are immunogenotypically and immunophenotypically heterogeneous. They may be derived from more than one lineage and/or different stages of differentiation. This heterogeneity may reflect the heterogeneity of the normal LGL population and is consistent with our preliminary observations that T_β gene rearrangement and expression is found in only a subpopulation of normal peripheral blood LGL cells (Pelicci PG et al, manuscript in preparation). With respect to the present study, we conclude that the large category of T-CLL may include a distinct subset of cases with LGL morphology, the monoclonal nature of which remains to be established.

Establishing the monoclonal nature of these cases bears important implications for their differential diagnosis, prognosis, and therapeutic management. First, it is important to note that the monoclonality of these diseases does not necessarily imply their complete malignancy. At least some of these cases may represent early, yet persisting stages of a multi-step leukemogenic process. The existence of cases that undergo a progressive change in their clinical course switching toward a more aggressive and malignant phenotype[2] supports this notion. Analysis of T_β gene rearrangements in longitudinal studies is likely to be useful in determining the derivation of these more malignant clones, by comparing their genotype and phenotype with those cells of earlier stages of the disease. The determination of clonality by T_β gene rearrangement analysis may be of significant diagnostic and prognostic value. Although our study suggests that even the most clinically chronic T-cell proliferations are of monoclonal origin, immunogenotypic analysis may still be needed to clarify difficult diagnostic situations. Once the determination of monoclonality has been made, the immunogenotypic marker would appear to represent a convenient tool with which to follow the biologic behavior and the therapeutic response of the pathologic clone, thus allowing improved monitoring of the patients' clinical course.

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CORRESPONDENCE

EPSTEIN-BARR GENOMES

To the Editor:

In the recent article, "Epstein-Barr Genomes are Restricted to Secondary Neoplastic Cells Following Bone Marrow Transplantation," by William H. Schubach, George Miller and E. Donnall Thomas,[1] the authors concluded that marrow transplant patients acquire Epstein-Barr virus (EBV) genomes infrequently in engrafted normal lymphocytes. From the title of Table 1 it is presumed that DNA samples were from peripheral blood mononuclear cells and that EBV genome was found in five patients. However, in the results section it is stated that the EBV genomes were contained in the cells of secondary lymphoid neoplasms. Did both the peripheral blood mononuclear cells as well as the neoplastic tumor cells contain EBV? If in these EBV associated tumor patients the peripheral blood mononuclear cells were found not to contain EBV, then perhaps the possibility exists that circulating mononuclear cells may not be a sensitive marker of the acquisition of EBV. Perhaps in the posttransplant setting, tissue such as lymph nodes or spleen might contain EBV genomes since these are sites for the development of secondary lymphoid neoplasms.[2] Did the authors study any other tissue besides peripheral blood mononuclear cells for EBV genome?

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To the Editor:

Dr Wolff asks the important question whether we sought EBV genomes in B cell repositories such as lymph nodes or spleen from patients not suffering from secondary lymphomas. For a variety of practical reasons this could not be done in the reported study. We chose to look for the acquisition of EBV genomes in bone marrow and peripheral blood at various times after engraftment. Aside from considerations of feasibility and convenience, this seemed a likely tissue to examine, since repopulation of marrow by B cells[1,2] of donor origin[3] has been documented by others. In these same studies, repopulating B cells were transformed in vitro by EBV. In our study we did not examine peripheral blood mononuclear cells of patients suffering secondary lymphomas. The EBV genomes found in these cases were detected in tumor samples, the only tissues examined from these patients. We do not know whether lymphocytes in other tissues contained EBV and we agree that such a study would be informative.

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EFFECT OF ALPHA AND GAMMA INTERFERON ON CFU-GM

To the Editor:

The conclusion of Rigby et al[1] that both alpha and gamma recombinant interferons inhibit granulocyte/monocyte colony-forming cells (CFU-GM) growth in a dose-dependent manner, a conclusion already reached by several groups,[2-4] is probably not compromised by the experimental approach used. However, for the other conclusions, it must be pointed out that the results were obtained in a culture system containing, as the source of colony stimulating activity, leukocyte-conditioned medium stimulated by 10% phytohemagglutinin (PHA) and normal blood mononuclear cells which had not been depleted of T lymphocytes. In a study designed to explore the effect of interferons on normal and leukemic cells as well, this type of stimulation of CFU-GM is open to criticism.

First, previous studies have shown that lymphocytes stimulated by PHA produce, in vitro and in vivo, sufficient amounts of gamma interferon to inhibit CFU-GM growth.[5,6] Thus, to conclude that gamma interferon is 10 to 100 times more cytotoxic to CFU-GM than alpha interferon, T lymphocyte-depleted blood cells should have been used instead of the whole mononuclear fraction to compare the normal and leukemic progenitors.

Second, PHA present in the culture medium is known to stimulate the growth of T lymphocyte colonies. Therefore, the observed effect of both alpha and gamma interferons might not be restricted to the CFU-GM but could instead reflect the inhibitory activity exerted on both CFU-GM and T lymphocyte colonies, since laboratory and clinical studies suggest that interferons inhibit T lymphocyte proliferation.[7,8] This is also true when the authors compare normal CFU-GM and blast progenitor cells. Thus, their conclusion that normal and leukemic myeloid progenitors are equally inhibited by the interferons appears debatable to us.

In our hands, with human placental conditioned medium as the colony stimulating activity and T-depleted mononuclear cells from leukemic patients stimulated by 10% PHA-LCM, we have observed in a limited number of cases (14 normal subjects, and three patients with acute nonlymphoblastic leukemic) that leukemic progenitors were less sensitive than normal CFU-GM to alpha and gamma recombinant interferon (Boehringer Ingelheim).

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To the Editor:

We are appreciative of the thoughtful comments by Delforge et al. With regard to their first point, it is possible that gamma, as well as alpha, interferon was present in our lymphocyte conditioned medium. Nevertheless, it is unlikely that significant differential effects resulted as linear dose-response curves were observed with each interferon. Furthermore, Broxmeyer et al reported similar data on day 14 granulocyte/monocyte colony-forming cells (CFU-GM) using cell line giant cell tumor (GCT) conditioned medium as a source of colony-stimulating activity, thus lacking any potential interferon contamination.[1]

Second, the colonies in our cultures were clearly CFU-GM in morphology and we have confirmed this through Wright-Giemsa staining. Leukemic colonies from donor 1 did not display typical CFU-GM morphology but, instead, were less well differentiated. Significant lymphocyte colony contamination seemed a most unlikely possibility, given that 100% of the patient's leukocytes were blasts.

Finally, we look forward to the evolution and publication of their preliminary studies on leukemic progenitor cells. Potential differences between their observations and ours may result as a consequence of their use of two sources of colony stimulating activity in their cultures, as well as the innate variability of myeloid leukemia cells.

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ANTI-COMMON ACUTE LYMPHOBLASTIC LEUKEMIA ANTIBODY (CALLA) (J5) REACTIVITY BY SMALL CELL LUNG CANCER (SCLC) CELLS

To the Editor:

In a recent article in *Blood*, Bunn et al[1] reported the expression of lymphoreticular antigens (Leu-7, Leu-11, OKT10, OKM1, Leu-M1, Leu-M2, OKT9) as detected by immunofluorescence analysis on cell lines deriving from oat cell carcinoma of the lung. In another article in *Science*, Ruff and Pert[2] describe the presence of OKM1 antigen on two SCLC cell lines and four SCLC autopsy specimens; they propose that the observed expression of OKM1 by SCLC may indicate the origin of SCLC from bone marrow macrophages; in contrast to the proposed theory, Gazdar et al[3] point out that shared antigenicity does not necessarily indicate common lineage or embryologic origin.

We are reporting here for the first time the binding of the J5 (anti-CALLA)[4] monoclonal antibody to SCLC cells deriving from a bone marrow specimen of a patient with lung, bone, and bone marrow involvement by SCLC at an advanced disease stage. Flow cytometric analysis of the SCLC cells stained by an indirect immunofluorescent technique showed that 16% of such cells were J5 positive. In addition, 41% of the small cells were Leu-M1 positive and 22% were Leu-9 positive. The Ia (HLA-DR related) antigen as well as kappa and lambda chains and the MO_2 antigen were not detected on the cell surface. Leukemic cells were not observed in the bone marrow smear or on biopsy specimen.

The J5 monoclonal antibody has also been reported to react with adult and fetal renal proximal tubule cells, adult breast myoepithelium[5] as well as common bile duct cells. Although CALLA antigenic expressions occur in cultured marrow and skin fibroblasts[6] there is not enough evidence to prove that SCLC derives from such ancestor cells. Mature granulocytes also express CALLA.[6] The biologic significance of such expression by normal or cultured cells remains to be proven.

We agree with Gadzar et al that caution should be applied in interpreting the shared reactivity of various tissues with white cell antibodies as adequate and as the sole indicator for a common embryologic origin. Expressions of such antigens by malignant cells may indicate gene dysregulation rather than a common origin, especially in highly undifferentiated tumors like SCLC.

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To the Editor:

We agree with the comments of Drs Efremidis and Bekesi regarding the interpretation of antigens shared by many tissues of diverse embryonic origin. We have found CALLA expressed on three of 13 small cell lines we have evaluated. The expression was noted in a minority of cells (38% to 50%) in the lines. We have previously reported low or absent expression of HLA class I antigens in small cell carcinoma.[1] In our experience, HLA-DR-related antigens are more normally expressed, but your findings are of interest. We continue to believe that small cell lung cancer is an epithelial tumor with neuroendocrine properties.[2,3]

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MYELOFIBROSIS WITH MYELOID METAPLASIA

To the Editor:

I have read with interest the paper of Wolf and Neiman entitled "Myelofibrosis with myeloid metaplasia: Pathophysiologic implications of the correlation between bone marrow changes and progression of splenomegaly" in the April issue of BLOOD (65:803, 1985). We recently had two boys (14 and 9 years old) with agnogenic myeloid metaplasia; in one this patchy appearance of stromal proliferation was well shown with augmented megakaryocytes by chance, supporting the authors' finding.[1]

Although the authors mentioned infection, metastatic carcinoma, exposure to myelotoxins and other hematologic disorders as a cause of secondary myelofibrosis, they did not refer to rickets, vitamin D deficiency,[2] or chronic renal failure.[3] I would like to bring into focus that increased bone marrow collagen, megakaryocytes and stromal hyperplasia all could be related to vitamin D metabolytes and/or increased parathormone level.[4]

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ANNOUNCEMENTS

CLINICAL HEMATOLOGY AND ONCOLOGY: 1986

February 17–19, 1986
Vacation Village Resort, San Diego, Calif
Sponsored by Scripps Clinic and Research Foundation, La Jolla, Calif.
Ernest Beutler, MD, Program Chairman

This course is designed for hematologists, oncologists, internists, pediatricians, and pathologists interested in the diagnosis and management of hematologic and oncologic disorders. An outstanding faculty from across the continent has been assembled to discuss various aspects of these fields, to aid clinicians in sorting out the important from the trivial, and to help identify those recent advances which will withstand the test of time.

For further information and application forms, contact: Bonny Mower, Department of Academic Affairs, Box 400S, Scripps Clinic and Research Foundation, 10666 N Torrey Pines Rd, La Jolla, CA 92037. (619) 457-8556.

The members of the International Society of Blood Transfusion (ISBT) Working Party on Terminology for Red Cell Surface Antigens wish to bring their Munich Report, (*Vox Sang* 49:171–175, 1985), to the attention of the readership of *Blood*.

The Working Party was established to promote uniformity and nonduplication in alphabetical nomenclature for red cell surface antigens and to provide a standard numerical terminology. The report contains listings of ISBT-approved designations and guidelines for the designations of "new" antigens. Interested persons may wish to contact local committee members: *Australia* (S. Young); *Canada* (M. Crookston, M. Lewis, B.P.L. Moore); *Denmark* (J. Jørgensen), *England* (D.J. Anstee, G.W.G. Bird, M. Contreras, G. Daniels, C.M. Giles, A. Lubenko, P.A. Tippett); *Finland* (H. Nevanlinna, P. Sistonen), *France* (Ch. Salmon); *Germany* (W. Dahr, S. Seidl); *Holland* (C.P. Engelfriet); *Japan* (Y. Okubo, J. Yasuda); *New Zealand* (P. Booth, G. Woodfield); *Norway* (L. Kornstad, R. Nordhagen); *USA* (F.H. Allen, Jr, E. Brodheim, P.D. Issitt, W.L. Marsh, P. Morel, J.J. Moulds, R.E. Rosenfield, B. Sabo, R.H. Walker).

The Neurovascular Society of North America/Society for Neurovascular Surgery requests abstracts dealing with acute or chronic neurologic manifestations due to a neurovascular, hemodynamic, or hematological mechanism. Any clinical aspect of a problem may be discussed. The NVSNA/SNVS meeting will be held on April 17–19, 1986 in Chicago. Abstract Deadline: January 15, 1986.

For further information contact Andrew L. Carney MD: call 1 (312) 482-3950 or write NVSNA/SNVS, Box 679, Oak Park, IL 60302.

HYBRIDOMAS IN BIOTECHNOLOGY AND MEDICINE

March 3–7, 1986
Boston, Mass.

Organizers: T.A. Springer, PhD, S.J. Burakoff, MD, E.L. Reinherz, MD
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This course is designed for scientific investigators interested in the latest innovations and implications of immunology and hybridoma technology. Monoclonal antibodies and T cell lines are extraordinarily specific and powerful reagents which have opened up new avenues of investigation in clinical medicine, biotechnology, and diverse biological disciplines. A distinguished faculty will review the relevant basic immunology and highlight the major advances achieved by application of this new technology. A comprehensive syllabus including selected methods protocols will be provided.

Ths course is divided into five sections: (1) Antibody diversity and monoclonal antibodies; (2) Applications of monoclonal antibodies in basic biomedical research; (3) Monoclonal antibodies in diagnosis and therapy; (4) Uses of antibodies in recombinant DNA technology; and (5) T cell clones, hybridomas, and their products.

To register and for additional information, write to Harvard Medical School, Department of Continuing Education, Boston, MA 02115 or call (617) 732-1525 from 10 AM to 4 PM.

Blood, Vol 67, No 1 (January), 1986: pp 254–256

TRANSPLANTATION SYMPOSIUM

April 28–30, 1986
Washington, DC

The American Red Cross announces its 1986 Annual Symposium, "Transplantation: Progress and Prospects." Emphasis will be on graft rejection and its prevention. Specific topic areas will be: Immunology of Graft Rejection— biology and function of T cells, role of humoral antibodies, review of experimental studies; Prevention of Rejection by Treatment of the Graft—class II antigen depletion, irradiation, T cell depletion, and immunosuppression in graft-*v*-host disease; Prevention of Rejection by Treatment of the Recipient—transfusion therapy, monoclonal antibodies, total lymphoid irradiation, and cyclosporine; Other Critical Aspects of Transplantation— reperfusion injury, organ perfusion, organ cryopreservation.

Attendance will be limited to 280. To receive further mailings, registration, and hotel information, write to Harold T. Meryman, MD, Biomedical Research and Development Laboratory, American Red Cross, 9312 Old Georgetown Rd, Bethesda, MD 20814.

INTERNATIONAL CONFERENCE ON AIDS

June 23–25, 1986
Palais des Congres, Paris

The congress will cover all aspects of contemporary research: virology, molecular biology, animal models, immunology, clinical aspects, pediatric AIDS, African AIDS, therapy, diagnostics, serology, epidemiology, public health, and psychosocial implications. Deadline for abstracts is February 1, 1986. For all information please contact Dr Jean-Claude Gluckman, Faculté de Médecine Pitié-Salpêtrière, 91 Boulevard de l'Hôpital, 75634 Paris Cedex 13, France. Telephone: (1) 45 70 27 02.

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17th INTERNATIONAL CONGRESS OF THE WORLD FEDERATION OF HEMOPHILIA

June 8–13, 1986
Universita Statale, Milan, Italy

The main theme of the Congress is *Progress in Hemophilia Through Biotechnology.* Main symposia are: The use of gene probes for prenatal diagnosis and carrier detection; production of clotting factors by biotechnologies; clinical trials of virus-inactivated concentrates; special diagnostic and clinical problems in hemophilia; the acquired immunodeficiency syndrome in 1986, an update; von Willebrand's disease; and the dentist and hemophilia.

Free communications and posters: Deadline for abstract presentation: Jan 31, 1986.

Scientific Secretariat: P.M. Mannucci, Via Pace 9, 20122 Milano, Italy. Telex: 315469 MGRMIL I.

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SEMINARS IN HEMATOLOGY

Editors

Peter A. Miescher, M.D., and
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A topical journal on subjects of current importance in clinical hematology and related fields, **Seminars in Hematology** is devoted to making the present status of such topics and the results of new investigations readily available to the practicing physician. It is of special interest to hematologists, oncologists, internal medicine specialists, blood bankers, and specialists in thrombosis and hemostasis.

SCHEDULED TOPICS, VOLUME 23, 1986:

JANUARY — PLATELETS AND MEGAKARYOCYTES IV
Guest Editors: *Paul K. Schick, M.D., and Helge Stormorken, M.D.*

APRIL — PLATELETS AND MEGAKARYOCYTES V
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POLYCYTHEMIA VERA: AN UPDATE
Guest Editor: *Nathaniel I. Berlin, M.D.*

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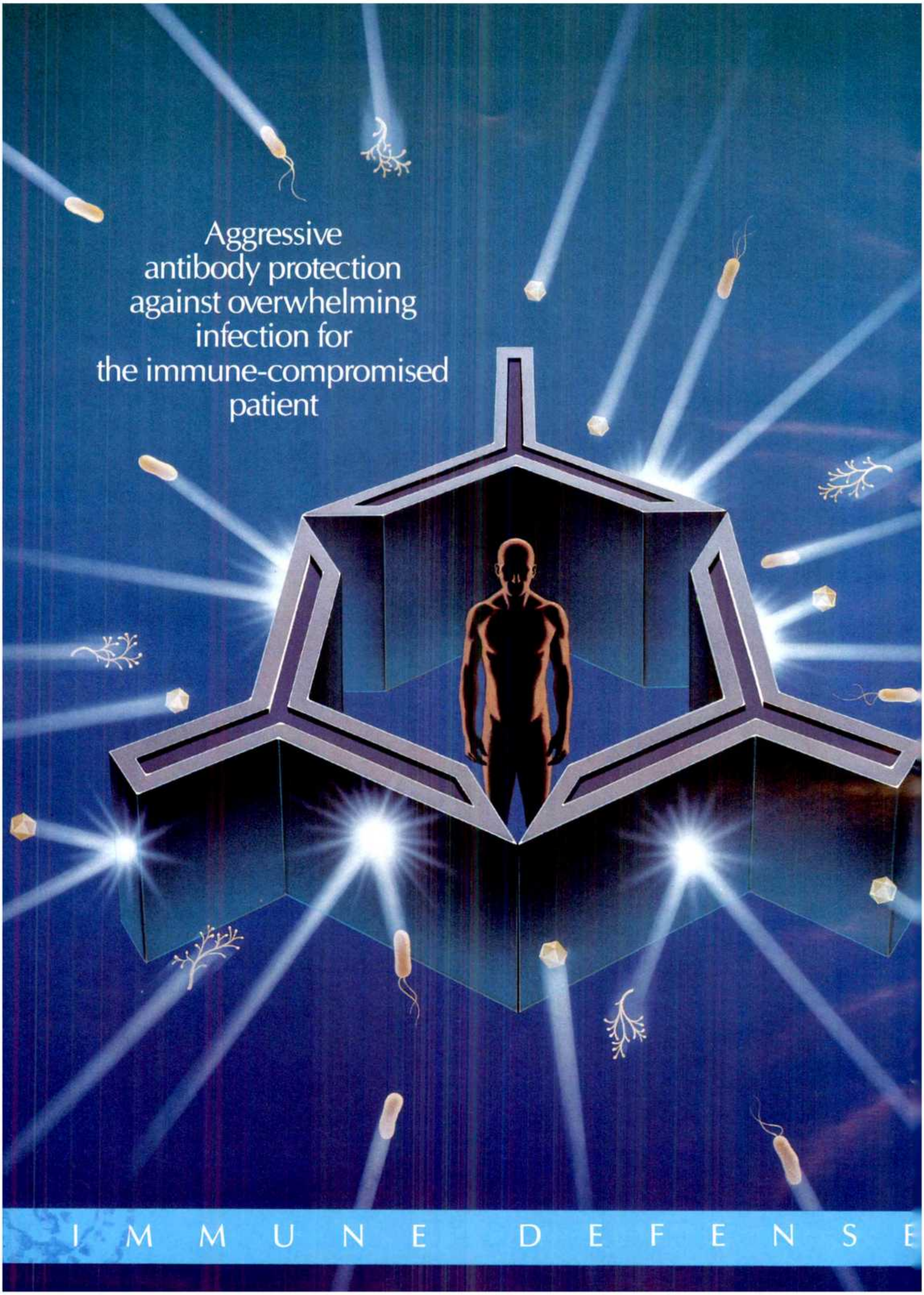
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(IFA method)	1:24	< 1:4			
			Parainfluenza		
Antistreptolysin-O	224IU	< 60IU	Type 1	1:8	< 1:8
			Type 2	1:8	< 1:8
H. influenzae Type B	1:14,700	1:1,600	Type 3	1:8	< 1:8
			Varicella-zoster	1:512	1:64
P. aeruginosa					
Lipopolysaccharide					
immunotype T-1	1:2,300	1:760	Cytomegalovirus		
T-2	1:7,100	1:1,070	(IFA method)	1:1,024	1:256
T-4	1:1,800	1:400	(IHA method)	1:2,048	1:512
T-6	1:1,800	1:600			
			P. carinii	1:4	< 1:4
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Type W 135 polysaccharide	1:8,400	1:1,600			

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Please see following page for brief summary of prescribing information.

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Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose)-Gamimune® is a sterile 5 ± 1% solution of human protein stabilized with 10 ± 2% maltose; it contains no preservatives. The immunoglobulin is selectively reduced under controlled conditions using dithiothreitol and alkylated with iodoacetamide to render it suitable for intravenous administration. Each 10 mL contains approximately 500 mg of protein, not less than 90% of which is immunoglobulin. Thus, there are approximately 450 mg of immunoglobulin in each 10 mL of IGIV, 5% Maltose. The pH is 5.25 ± 0.25. The calculated osmolality is 327 milliosmoles per kilogram of solvent (water) and the calculated osmolarity is 292 milliosmoles per liter of solution. The product is prepared by cold alcohol fractionation from large pools of human venous plasma. Each individual unit of plasma has been tested and found nonreactive for hepatitis B surface antigen (HBsAg) using a test with third-generation sensitivity.

CLINICAL PHARMACOLOGY

Gamimune supplies IgG antibodies for prevention or attenuation of infectious diseases. The half-life of Gamimune is approximately three weeks but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immunoglobulin administered per dose is important in determining the frequency of administration of the drug for each patient.

While intravenous immunoglobulin has been shown to be effective in some cases of idiopathic thrombocytopenic purpura (ITP) (see Indications and Usage), the mechanism of action in ITP has not been fully elucidated.

Maltose is added to the solution in a 10% concentration for stabilization of the protein. A comparative clinical study of IGIV with and without maltose showed the incidence of adverse effects was significantly less with the maltose-containing preparation.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well and no adverse effects were observed at a rate of 0.25 g maltose/kg per hour. In safety studies conducted by Cutter Laboratories, infusions of 10% maltose administered at 0.27 to 0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous infusions of maltose, maltose was detected in the peripheral blood, and there was a dose dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were tolerated without significant adverse effects.

Since one milliliter of Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose)-Gamimune® contains 0.1 g of maltose, the recommended dose of Gamimune (0.1 g to 0.4 g per kilogram body weight) would result in the patient receiving a total of 0.2 g to 0.8 g maltose per kilogram body weight given over several hours. These amounts are within those levels tolerated in the Cutter safety studies cited above.

INDICATIONS AND USAGE

Immunodeficiency Gamimune is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune may be preferred to that of intramuscular immunoglobulin preparations especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., x-linked agammaglobulinemia), common variable hypogammaglobulinemia, x-linked immunodeficiency with hyper IgM, and combined immunodeficiency.

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Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose)-Gamimune® is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Serum Globulin (Human). Individuals with selective IgA deficiencies who have known anti IgA antibody should not receive IGIV, 5% Maltose since these patients may experience severe anaphylactic reactions to even small amounts of immune globulin preparations.

WARNINGS

IGIV, 5% Maltose should be administered only intravenously as the intramuscular route has not yet been evaluated.

> IGIV, 5% Maltose can, on occasion, cause a precipitous fall in blood pressure and the clinical picture of anaphylaxis even when the patient is not known to be sensitive to immune globulin preparations. These reactions appear to be related to the rate of infusion. Accordingly, the infusion rate given under Dosage and Administration should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for treatment of any acute anaphylactoid reaction.

PRECAUTIONS

General Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid.

Drug Interactions If dilution is required Gamimune may be diluted with 5% dextrose. No other drug interactions or compatibilities have been evaluated. It is recommended that the infusion of Gamimune be given via separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C Animal reproduction studies have not been conducted with Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose)-Gamimune®. It is also not known whether Gamimune can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune should be given to a pregnant woman only if clearly needed.

Pediatric Use High dose administration of Gamimune in children with idiopathic thrombocytopenic purpura (ITP) did not reveal any adverse effects specific to the pediatric age group.

ADVERSE REACTIONS

In a small safety study of Gamimune in ten patients with primary immunodeficiency diseases, who received a total of 28 infusions at a dose of 400 mL per kg body weight, there were no adverse reactions reported during any of the infusions. Post infusion, three patients reported adverse effects "possibly" or "definitely" related to the IGIV infusion; two patients experienced post infusion headache, and one had a flu-like syndrome consisting of joint pain, red eyes, and fatigue following the infusion.

During additional investigational studies, 17 other patients with idiopathic thrombocytopenic purpura (ITP) received a total of 148 infusions of Gamimune. Most of these infusions were at doses of 400 mg/kg body weight for 5 consecutive days; however, some patients received either 400 mg/kg or 800 mg/kg body weight as a single infusion intermittently. In these studies, adverse reactions were reported in 17 of 148 (11.5%) infusions of Gamimune. Headache, generally mild, was the most common symptom noted, occurring during or following 8% of infusions. Other less frequently reported symptoms included emesis, chills, fever, chest tightness, and nausea. With rare exceptions, symptoms were either mild or moderate in degree.

Potential reactions with administration of intravenous gamma globulins may also include anxiety, flushing, chills, wheezing, abdominal cramps, back pain, myalgia, arthralgia, malaise and dizziness; rash has been reported only rarely. These reactions tend to be related to the rate of infusion; their management is described under Dosage and Administration.

True anaphylactic reactions to IGIV, 5% Maltose may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immune serum globulin, but some patients may tolerate cautiously administered IGIV without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immune globulin.

DOSAGE AND ADMINISTRATION

Prophylaxis of Immunodeficiency Syndromes: The usual dose of Gamimune for prophylaxis in immunodeficiency syndromes is 100 mg/kg body weight (i.e., 2 mL/kg body weight) administered once a month by intravenous infusion. If the clinical response is inadequate or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be increased to 200 mg/kg body weight (i.e., 4 mL/kg body weight) or the infusion may be repeated more frequently than once a month.

Therapy of Idiopathic Thrombocytopenic Purpura (ITP): The recommended dosage of Gamimune for therapy of ITP is 400 mg/kg of body weight (i.e., 8 mL/kg body weight) on five consecutive days.

Administration: Recent investigations confirm that Gamimune is well tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune be infused, by itself, at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes. If the patient does not experience any discomfort, the rate may be gradually increased to between 0.02 and 0.04 mL/kg body weight per minute. If side effects occur, the rate should be reduced or the infusion interrupted until the symptoms subside. The infusion may then be resumed at a rate which is tolerated by the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit.

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(Continued on following page)

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The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which work was done; (4) acknowledgments for research support; (5) name and address of the author to whom communications regarding the manuscript should be directed.

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Personal communications and references to publications in press by authors other than those submitting the paper must be substantiated by a letter from the investigator(s) concerned confirming the data or observations and granting the authors permission to cite the material.

EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

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2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

Complete book:

3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

Chapter of book:

4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): Gamma Globulins, Proceedings of the Third Nobel Symposium. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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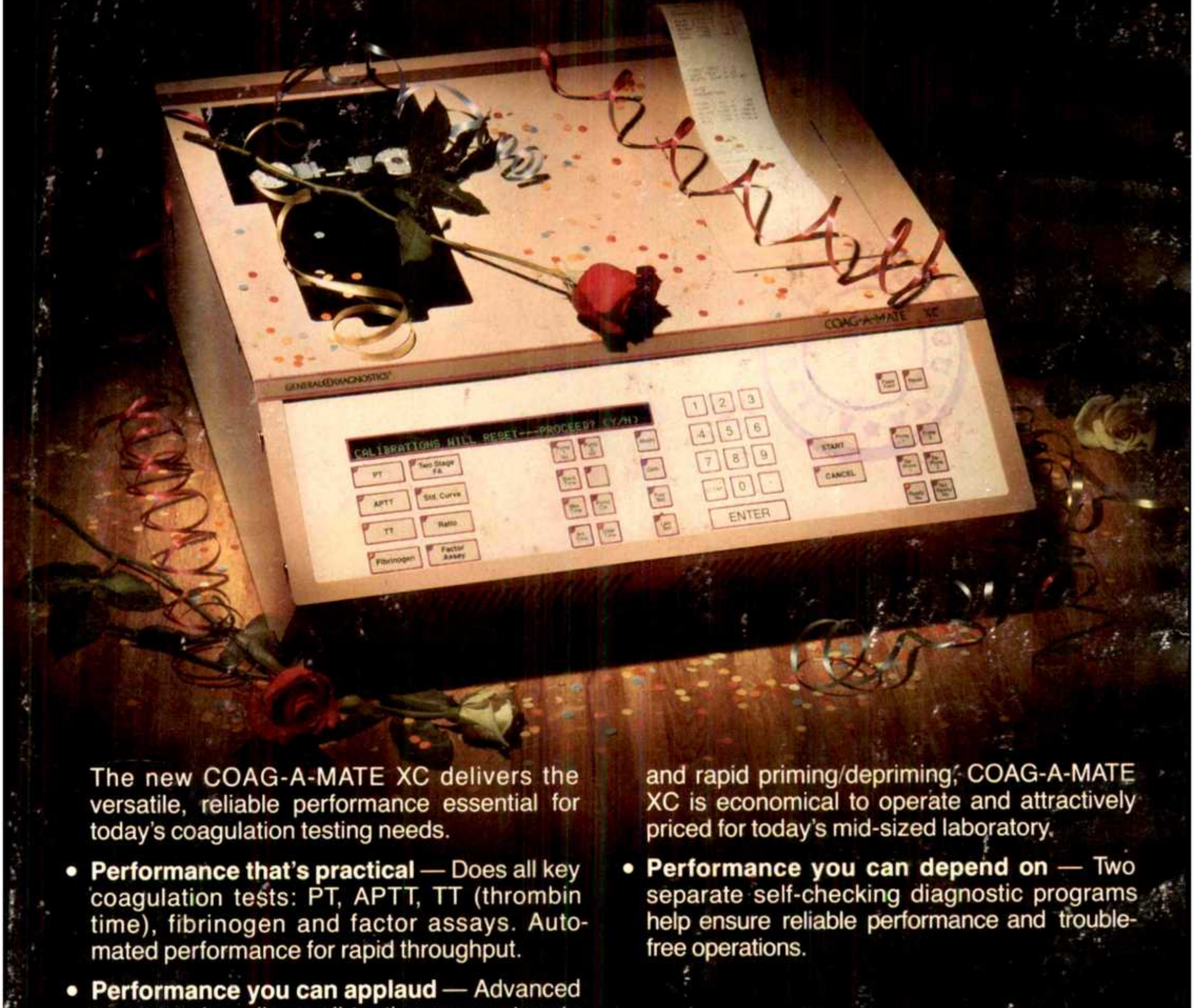

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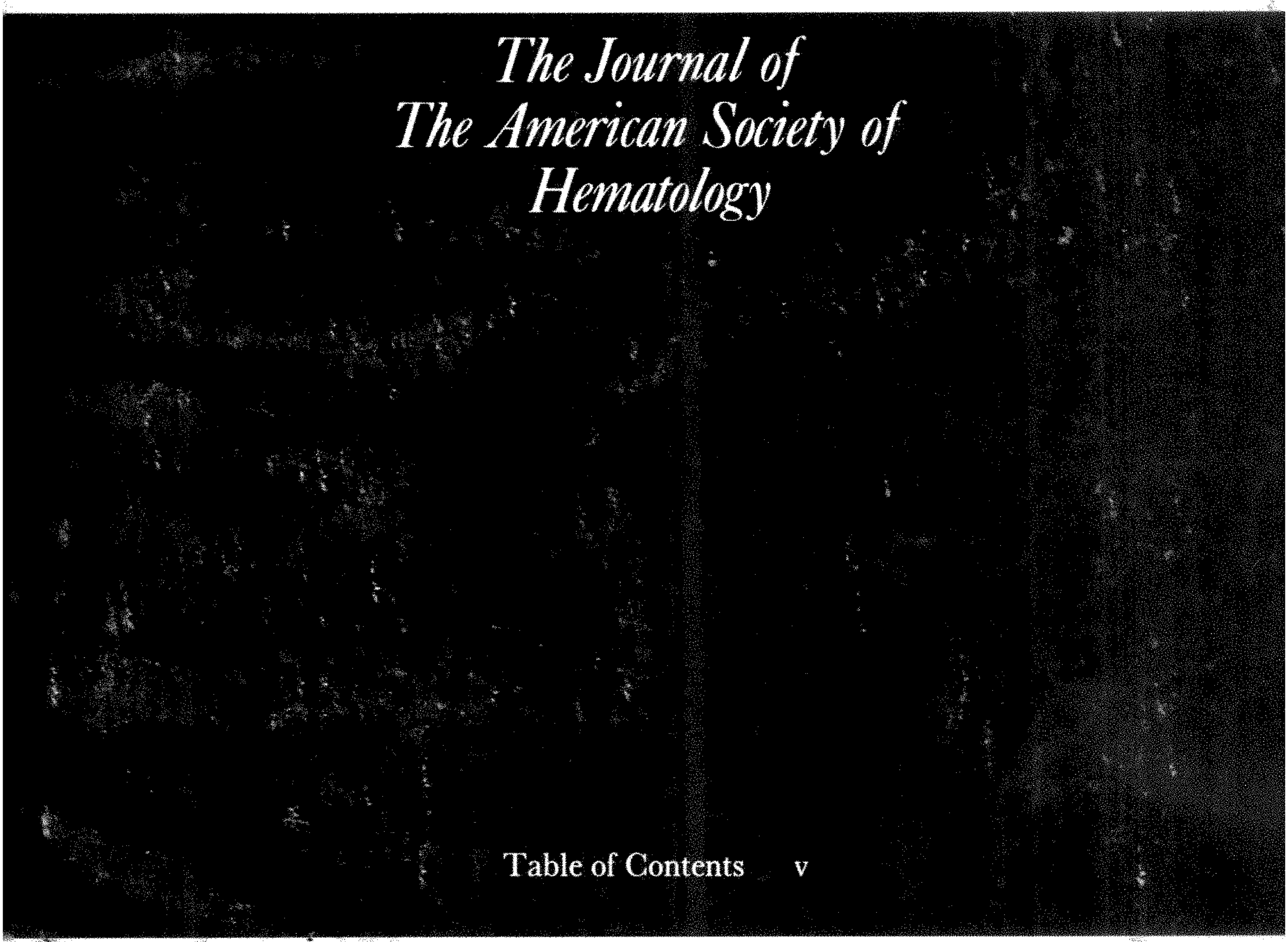

The Journal of The American Society of Hematology

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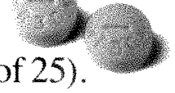

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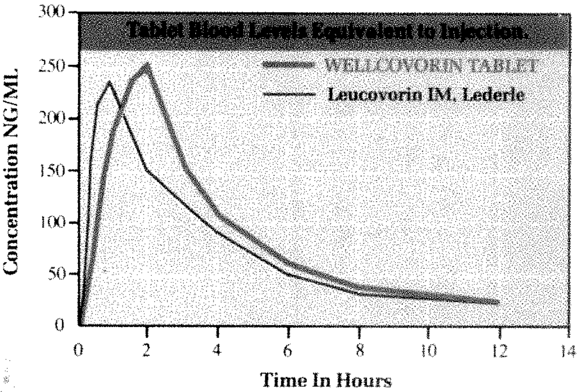
Blum MR, Guaspari A, Liao SHT, et al. Bioavailability and pharmacokinetics of oral and parenteral leucovorin. 33rd National Meeting Pharm Sci, San Diego, California, 1982, 12(2):175



Average serum folates after single 15 mg dose of leucovorin administered either IM or orally.
Adapted from Blum, et al

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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

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2. Frei E, Blum RH, Pitman SW, *et al:* High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med, 68*:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro. Br J Haematol, 40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al:* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis. 135*(3): 493-496, 1977.

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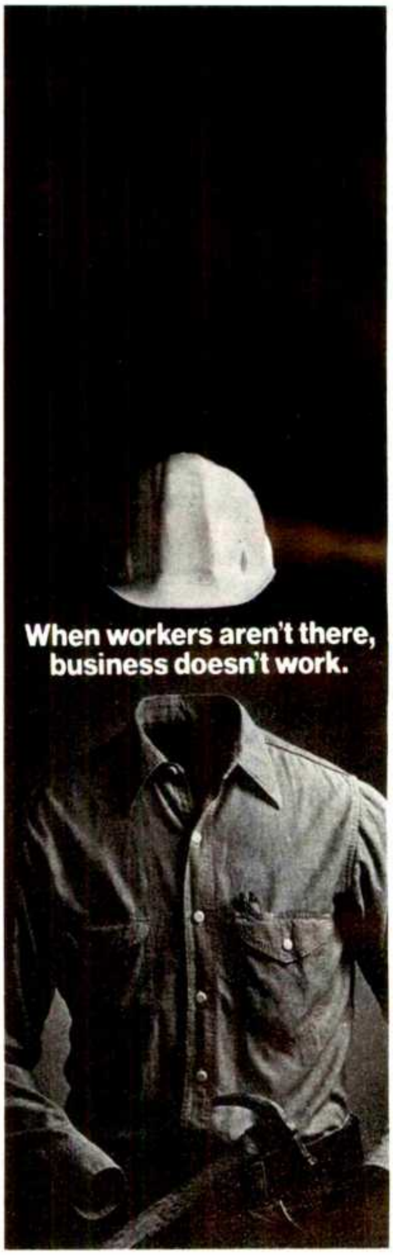

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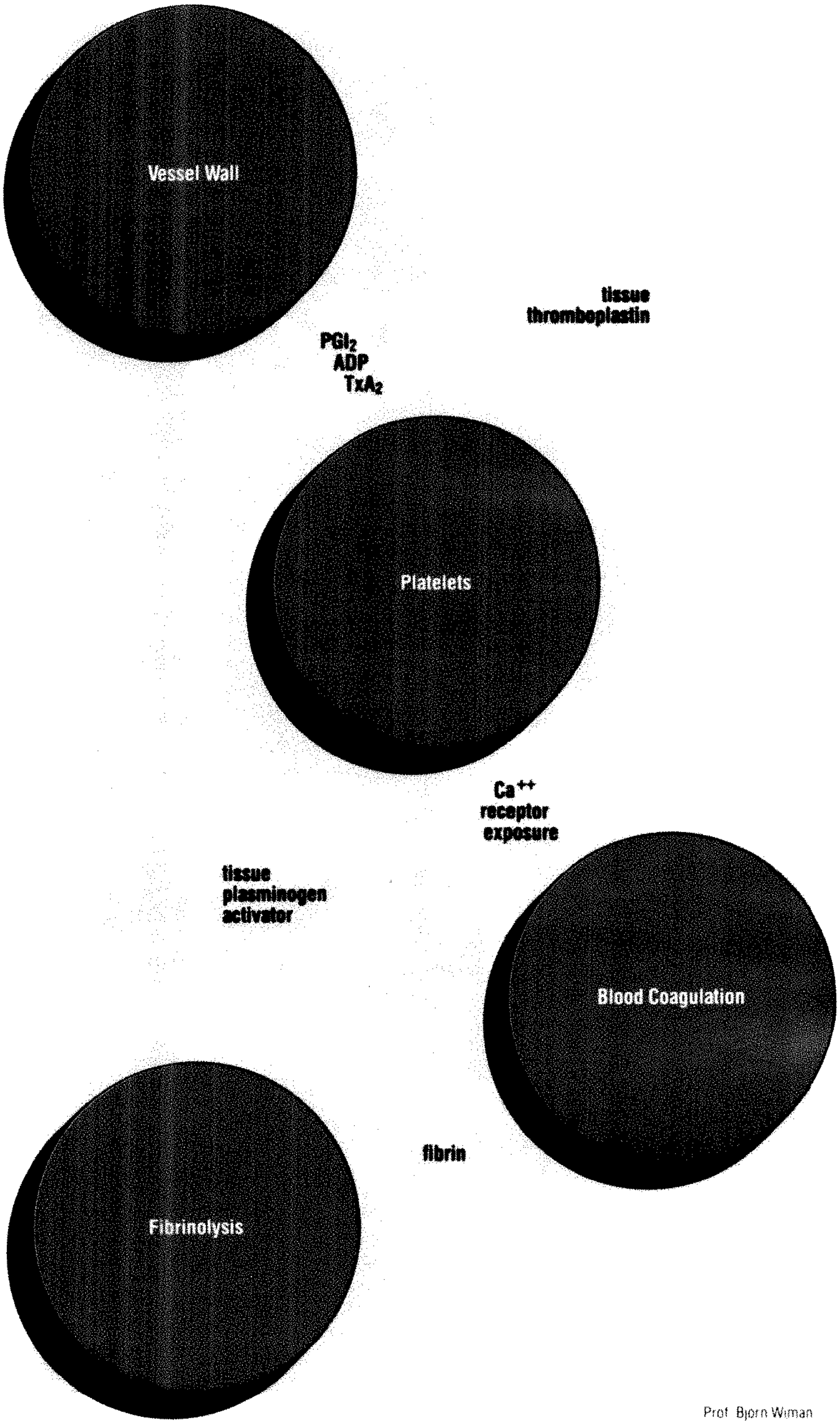

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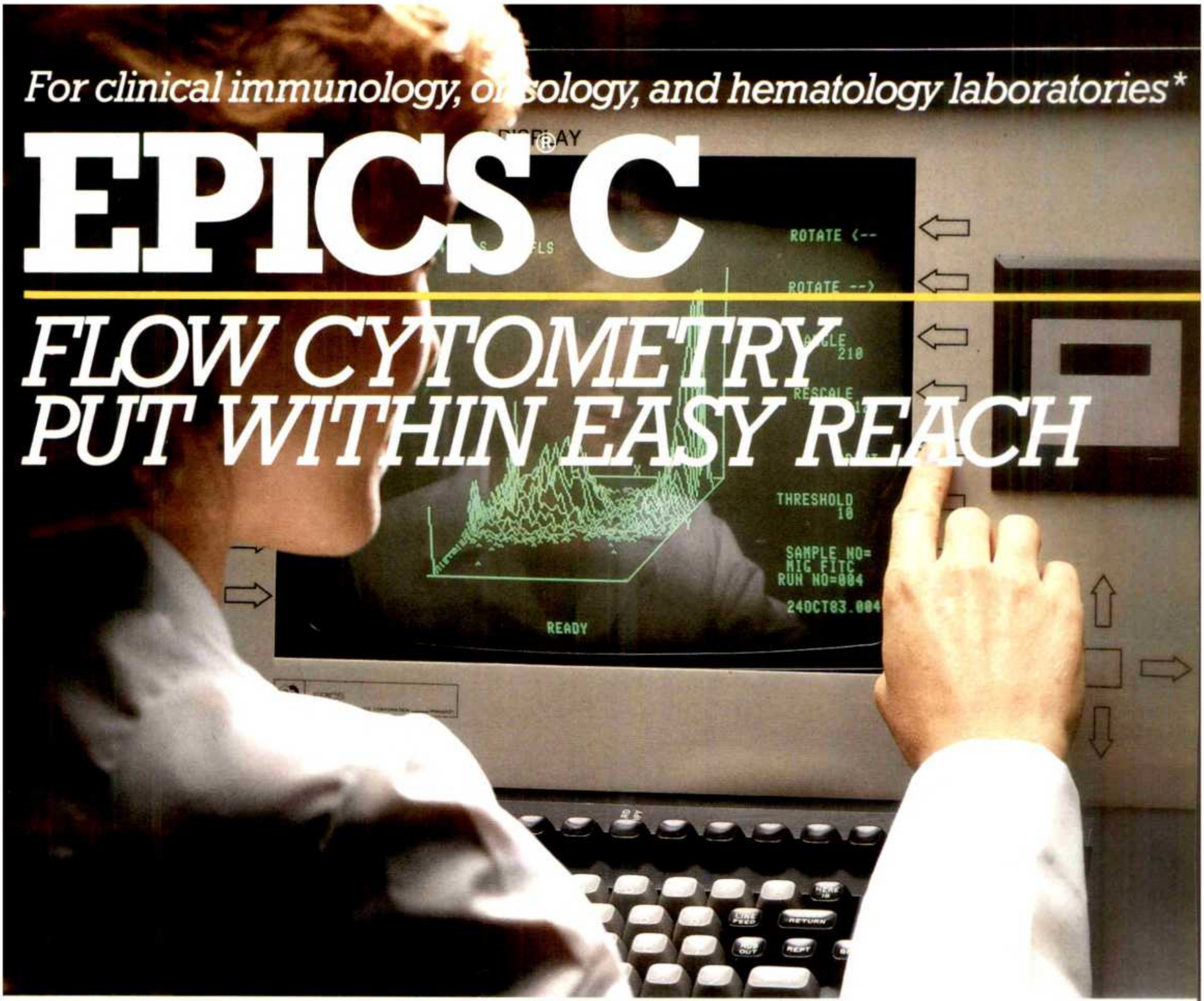

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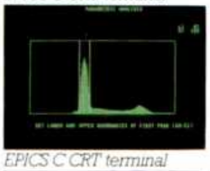

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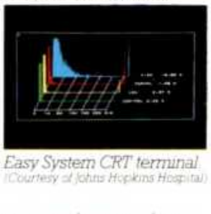

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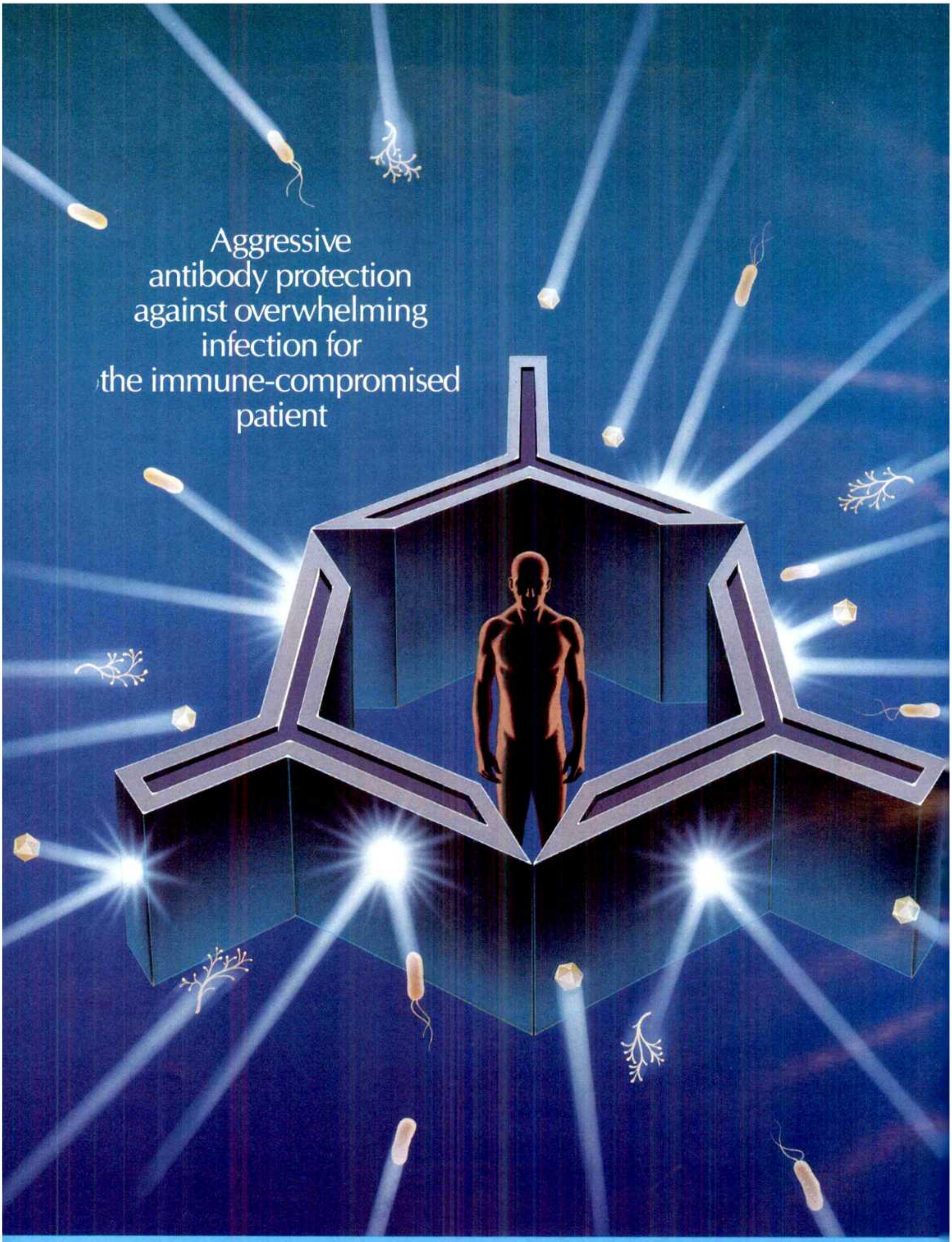
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(IHA method)	1:24	<1:4			
			Parainfluenza		
Antistreptolysin-O	224IU	<60IU	Type 1	1:8	<1:8
			Type 2	1:8	<1:8
H. influenzae Type B	1:14,700	1:1,600	Type 3	1:8	<1:8
			Varicella-zoster	1:512	1:64
P. aeruginosa					
Lipopolysaccharide					
immunotype T-1	1:2,300	1:760	Cytomegalovirus		
T-2	1:7,100	1:1,070	(IFA method)	1:1,024	1:256
T-4	1:1,800	1:400	(IHA method)	1:2,048	1:512
T-6	1:1,800	1:600			
			P. carinii	1:4	<1:4
N. meningitidis					
Type A polysaccharide	1:3,200	1:400			
Type C polysaccharide	1:8,400	1:800			
Type Y polysaccharide	1:1,400	1:200			
Type W 135					
polysaccharide	1:8,400	1:1,600			

* These values represent the geometric mean titers of 5 lots of GAMIMUNE

† These values represent a pool from at least 1,000 carefully screened donors

References:
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Please see following page for brief summary of prescribing information.

FROM WITHIN

GAMIMUNE®

Immune Globulin Intravenous, 5% (In 10% maltose)

DESCRIPTION

Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose) Gamimune® is a sterile 5 ± 1% solution of human protein stabilized with 10 ± 2% maltose; it contains no preservatives. The immunoglobulin is selectively reduced under controlled conditions using dithiothreitol and alkylated with iodoacetamide to render it suitable for intravenous administration. Each 10 mL contains approximately 500 mg of protein, not less than 90% of which is immunoglobulin. Thus, there are approximately 450 mg of immunoglobulin in each 10 mL of IGIV, 5% Maltose. The pH is 5.25 ± 0.25. The calculated osmolality is 327 milliosmoles per kilogram of solvent (water) and the calculated osmolarity is 292 milliosmoles per liter of solution. The product is prepared by cold alcohol fractionation from large pools of human venous plasma. Each individual unit of plasma has been tested and found nonreactive for hepatitis B surface antigen (HBsAg) using a test with third-generation sensitivity.

CLINICAL PHARMACOLOGY

Gamimune supplies IgG antibodies for prevention or attenuation of infectious diseases. The half-life of Gamimune is approximately three weeks but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immunoglobulin administered per dose is important in determining the frequency of administration of the drug for each patient.

While intravenous immunoglobulin has been shown to be effective in some cases of idiopathic thrombocytopenic purpura (ITP) (see Indications and Usage), the mechanism of action in ITP has not been fully elucidated.

Maltose is added to the solution in a 10% concentration for stabilization of the protein. A comparative clinical study of IGIV with and without maltose showed the incidence of adverse effects was significantly less with the maltose-containing preparation.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well and no adverse effects were observed at a rate of 0.25 g maltose/kg per hour. In safety studies conducted by Cutter Laboratories, infusions of 10% maltose administered at 0.27 to 0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous infusions of maltose, maltose was detected in the peripheral blood, and there was a dose dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were tolerated without significant adverse effects.

Since one milliliter of Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose) Gamimune® contains 0.1 g of maltose, the recommended dose of Gamimune (0.1 g to 0.4 g per kilogram body weight) would result in the patient receiving a total of 0.2 g to 0.8 g maltose per kilogram body weight given over several hours. These amounts are within those levels tolerated in the Cutter safety studies cited above.

INDICATIONS AND USAGE

Immunodeficiency Gamimune is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune may be preferred to that of intramuscular immunoglobulin preparations especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., x-linked agammaglobulinemia), common variable hypogammaglobulinemia, x-linked immunodeficiency with hyper IgM, and combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura Some children and adults with ITP have shown an increase (albeit temporary) in platelet counts upon administration of Gamimune. Therefore administration of Gamimune should be considered in situations that require a rapid, temporary rise in the platelet count, for example, prior to surgery or to control excessive bleeding. However, it should be noted that not all patients will respond. Even in those patients who do respond, this treatment should not be considered to be curative.

CONTRAINDICATIONS

Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose) Gamimune® is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Serum Globulin (Human). Individuals with selective IgA deficiencies who have known anti-IgA antibody should not receive IGIV, 5% Maltose since these patients may experience severe anaphylactic reactions to even small amounts of immune globulin preparations.

WARNINGS

IGIV, 5% Maltose should be administered only intravenously as the intramuscular route has not yet been evaluated.

> IGIV, 5% Maltose can, on occasion, cause a precipitous fall in blood pressure and the clinical picture of anaphylaxis even when the patient is not known to be sensitive to immune globulin preparations. These reactions appear to be related to the rate of infusion. Accordingly, the infusion rate given under Dosage and Administration should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for treatment of any acute anaphylactoid reaction.

PRECAUTIONS

General Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid.

Drug Interactions If dilution is required Gamimune may be diluted with 5% dextrose. No other drug interactions or compatibilities have been evaluated. It is recommended that the infusion of Gamimune be given via separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C Animal reproduction studies have not been conducted with Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose) Gamimune®. It is also not known whether Gamimune can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune should be given to a pregnant woman only if clearly needed.

Pediatric Use High dose administration of Gamimune in children with idiopathic thrombocytopenic purpura (ITP) did not reveal any adverse effects specific to the pediatric age group.

ADVERSE REACTIONS

In a small safety study of Gamimune in ten patients with primary immunodeficiency diseases, who received a total of 28 infusions at a dose of 400 mL per kg body weight, there were no adverse reactions reported during any of the infusions. Post infusion, three patients reported adverse effects "possibly" or "definitely" related to the IGIV infusion; two patients experienced post-infusion headache, and one had a flu like syndrome consisting of joint pain, red eyes, and fatigue following the infusion.

During additional investigational studies, 17 other patients with idiopathic thrombocytopenic purpura (ITP) received a total of 148 infusions of Gamimune. Most of these infusions were at doses of 400 mg/kg body weight for 5 consecutive days; however, some patients received either 400 mg/kg or 800 mg/kg body weight as a single infusion intermittently. In these studies, adverse reactions were reported in 17 of 148 (11.5%) infusions of Gamimune. Headache, generally mild, was the most common symptom noted, occurring during or following 8% of infusions. Other less frequently reported symptoms included emesis, chills, fever, chest tightness, and nausea. With rare exceptions, symptoms were either mild or moderate in degree.

Potential reactions with administration of intravenous gamma globulins may also include anxiety, flushing, chills, wheezing, abdominal cramps, back pain, myalgia, arthralgia, malaise and dizziness; rash has been reported only rarely. These reactions tend to be related to the rate of infusion; their management is described under Dosage and Administration.

True anaphylactic reactions to IGIV, 5% Maltose may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immune serum globulin, but some patients may tolerate cautiously administered IGIV without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immune globulin.

DOSAGE AND ADMINISTRATION

Prophylaxis of Immunodeficiency Syndromes: The usual dose of Gamimune for prophylaxis in immunodeficiency syndromes is 100 mg/kg body weight (i.e., 2 mL/kg body weight) administered once a month by intravenous infusion. If the clinical response is inadequate or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be increased to 200 mg/kg body weight (i.e., 4 mL/kg body weight) or the infusion may be repeated more frequently than once a month.

Therapy of Idiopathic Thrombocytopenic Purpura (ITP): The recommended dosage of Gamimune for therapy of ITP is 400 mg/kg of body weight (i.e., 8 mL/kg body weight) on five consecutive days.

Administration: Recent investigations confirm that Gamimune is well tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune be infused, by itself, at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes. If the patient does not experience any discomfort, the rate may be gradually increased to between 0.02 and 0.04 mL/kg body weight per minute. If side effects occur, the rate should be reduced or the infusion interrupted until the symptoms subside. The infusion may then be resumed at a rate which is tolerated by the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit.

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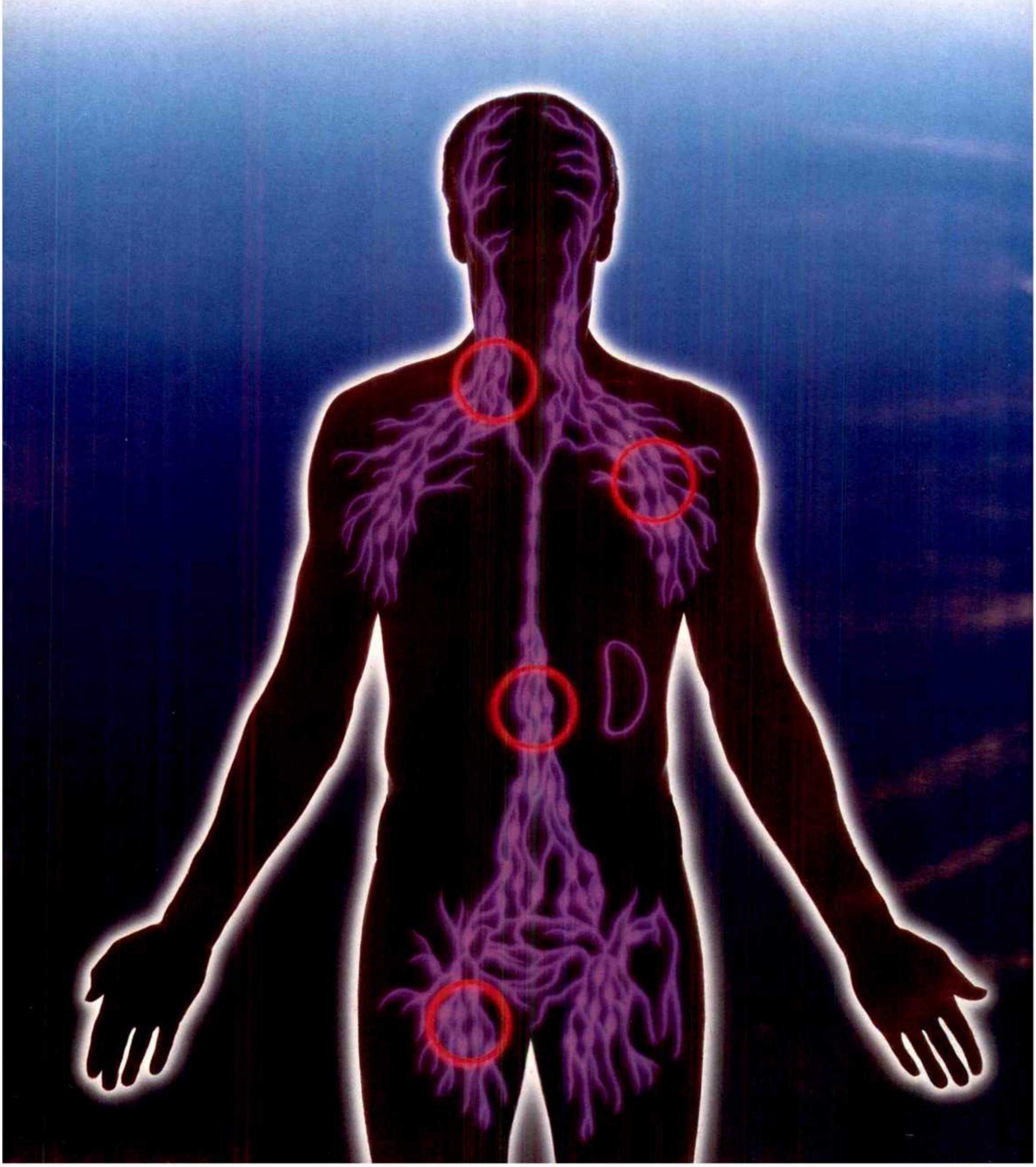
Aggressive Therapy for



Aggressive Lymphomas

Approaching a cure for non-Hodgkin's lymphomas (NHL)

Lymphomas were considered incurable a scant two decades ago; now, cure is a reality for as many as half of all patients with NHL.[1] The frequency of complete remissions—the most critical factor influencing prognosis—has increased, and long-term disease-free survival is an attainable goal for many patients.

With innovative therapeutic programs

Combination chemotherapy now serves as a primary therapy for the vast majority of patients with intermediate-to-poor prognosis NHL. Treatment strategies depend on many factors, including age, histology, and stage of the primary disease, and presence of comorbid disease. Consequently, no one therapeutic program can be cited as best. Fortunately, the availability of multiple non-cross-resistant regimens has permitted individualization of therapy.

Methotrexate...a component of aggressive regimens improving the outcome in NHL

Lederle introduced Methotrexate in the 1950s. Since then, Methotrexate has been used in many different ways, reflecting the changing approaches to clinical oncology. One important application is in the treatment of NHL.

1. DeVita VT Jr, Hellman S: Hodgkin's disease and the non-Hodgkin's lymphomas, in DeVita VT Jr, Hellman S, and Rosenberg SA (eds): *Cancer: Principles and Practice of Oncology.* Philadelphia, J.B. Lippincott Company, 1982, p. 1392.

Please review the brief summary of prescribing information on the next page

LEDERLE
METHOTREXATE

METHOTREXATE
Brief Summary

WARNINGS:

WARNING
METHOTREXATE MUST BE USED ONLY BY PHYSICIANS EXPERIENCED IN ANTIMETABOLITE CHEMOTHERAPY. BECAUSE OF THE POSSIBILITY OF FATAL OR SEVERE TOXIC REACTIONS, THE PATIENT SHOULD BE FULLY INFORMED BY THE PHYSICIAN OF THE RISKS INVOLVED AND SHOULD BE UNDER HIS CONSTANT SUPERVISION. DEATHS HAVE BEEN REPORTED WITH THE USE OF METHOTREXATE IN THE TREATMENT OF PSORIASIS.

IN THE TREATMENT OF PSORIASIS, METHOTREXATE SHOULD BE RESTRICTED TO SEVERE RECALCITRANT, DISABLING, PSORIASIS WHICH IS NOT ADEQUATELY RESPONSIVE TO OTHER FORMS OF THERAPY, BUT ONLY WHEN THE DIAGNOSIS HAS BEEN ESTABLISHED, AS BY BIOPSY AND/OR AFTER DERMATOLOGIC CONSULTATION.

1. Methotrexate may produce marked depression of bone marrow, anemia, leukopenia, thrombocytopenia, and bleeding.
2. Methotrexate may be hepatotoxic, particularly at high dosage or with prolonged therapy. Liver atrophy, necrosis, cirrhosis, fatty changes, and periportal fibrosis have been reported. Since changes may occur without previous signs of gastrointestinal or hematologic toxicity, it is imperative that hepatic function be determined prior to initiation of treatment and monitored regularly throughout therapy. Special caution is indicated in the presence of preexisting liver damage or impaired hepatic function. Concomitant use of other drugs with hepatotoxic potential (including alcohol) should be avoided.
3. Methotrexate has caused fetal death and/or congenital anomalies; therefore, it is not recommended in women of childbearing potential unless there is appropriate medical evidence that the benefits can be expected to outweigh the considered risks. Pregnant psoriatic patients should not receive Methotrexate.
4. Impaired renal function is usually a contraindication.
5. Diarrhea and ulcerative stomatitis are frequent toxic effects and require interruption of therapy; otherwise hemorrhagic enteritis and death from intestinal perforation may occur.

METHOTREXATE HAS BEEN ADMINISTERED IN VERY HIGH DOSAGE FOLLOWED BY LEUCOVORIN RESCUE IN EXPERIMENTAL TREATMENT OF CERTAIN NEOPLASTIC DISEASES. THIS PROCEDURE IS INVESTIGATIONAL AND HAZARDOUS.

INDICATIONS: Antineoplastic Chemotherapy: Methotrexate is indicated for treatment of gestational choriocarcinoma, in patients with chorioadenoma destruens and hydatidiform mole; palliation of acute lymphocytic leukemia, treatment and prophylaxis of meningeal leukemia, and palliation of acute lymphoblastic (stem-cell) leukemias in children. May be used for induction of remission in combination with other anticancer drugs or suitable agents, but is most commonly used in maintenance of induced remissions. May be used alone or in combination with other anticancer agents in management of breast cancer, epidermoid cancers of the head and neck, and lung cancer, particularly squamous cell and small cell types. Also effective in treatment of advanced stages of lymphosarcoma, particularly in children, and in advanced cases of mycosis fungoides. See box warnings re *Psoriasis Chemotherapy*, because of high risk Methotrexate is indicated only in symptomatic control of severe, recalcitrant, disabling psoriasis which is not adequately responsive to other forms of therapy, *only when diagnosis has been established, as by biopsy and/or after dermatologic consultation.*
CONTRAINDICATIONS: Psoriatic patients who are pregnant, or have severe renal or hepatic disorders; or have preexisting blood dyscrasias such as bone marrow hypoplasia, leukopenia, thrombocytopenia, or anemia, should not receive Methotrexate.
PRECAUTIONS: Methotrexate has high potential toxicity, usually dose-related; physicians should be familiar with various characteristics and its established clinical usage. Subject patients to appropriate supervision to detect and evaluate signs or symptoms of possible toxic effects or adverse reactions with minimal delay. Hematopoietic suppression is a common effect of Methotrexate; pretreatment and periodic hematologic studies are essential to its use in chemotherapy. This effect may occur abruptly and on apparently safe dosage; any profound drop in blood-cell count indicates immediate stopping of drug and appropriate therapy. Use drug with caution, if at all, in patients with malignant disease who have preexisting bone marrow aplasia, leukopenia, thrombocytopenia, or anemia.

Methotrexate is excreted principally by the kidneys; use in the presence of impaired renal function may result in accumulation of toxic amounts or additional renal damage. Renal status should be determined prior to and during therapy; exercise proper caution should significant renal impairment be disclosed. Reduce or discontinue drug dosage until renal function is improved or restored.

The following laboratory tests are recommended as part of essential clinical evaluation and appropriate monitoring of patients to determine any existing organ dysfunction or system impairment: complete hemogram, hematocrit, urinalysis, renal function and liver function tests, and chest x-ray. Perform tests prior to, at appropriate periods during, and after termination of therapy. Where high dose or long-term therapy is followed, performance of liver biopsy or bone marrow aspiration studies may be useful or important. Methotrexate is bound in part to serum albumin after absorption; toxicity may be increased because of displacement by drugs such as salicylates, sulfonamides, diphenylhydantoin, phenylbutazone; by antibacterials such as tetracycline, chloramphenicol and para-amino-benzoic acid. Do not give these drugs concurrently, especially salicylates, phenylbutazone, and sulfonamides, whether antibacterial, hypoglycemic or diuretic, until significance of these findings is established. Vitamin preparations containing folic acid or its derivatives may alter responses to Methotrexate. Use with extreme caution in presence of infection, peptic ulcer, ulcerative colitis, debility, and extreme youth or old age. Bacterial infection may occur or become a threat if profound leukopenia occurs during therapy; cessation of drug and appropriate antibiotic therapy is usually indicated. Blood or platelet transfusions may be necessary in severe bone marrow depression. Methotrexate may have an immunosuppressive action; take into consideration when immune responses in a patient may be important or essential. In all instances, physician must evaluate need and usefulness of drug against risk of toxic effects or adverse reaction; most adverse reactions are reversible if detected early. Reduce dosage or discontinue drug when such effects or reactions do occur; take appropriate corrective measures according to clinical judgement of physician. Reinstitute therapy with caution, consideration of further need for drug, and alertness to possible toxicity recurrence.
ADVERSE REACTIONS: Most common include ulcerative stomatitis, leukopenia, nausea and abdominal distress. Others reported are malaise, undue fatigue, chills and fever, dizziness and decreased resistance to infection. Incidence and severity of side effects are generally considered dose-related. *Skin*: erythematous rashes, pruritus, urticaria, photosensitivity, depigmentation, alopecia, ecchymosis, telangiectasia, acne, furunculosis. Concomitant exposure to ultraviolet radiation may aggravate lesions of psoriasis. *Blood*: bone marrow depression, leukopenia, thrombocytopenia, anemia, hypogammaglobulinemia, hemorrhage from various sites, septicemia. *Alimentary System*: gingivitis, pharyngitis, stomatitis, anorexia, vomiting, diarrhea, hematemesis, melena, gastrointestinal ulceration and bleeding, enteritis, hepatic toxicity resulting in acute liver atrophy, necrosis, fatty metamorphosis, periportal fibrosis, or hepatic cirrhosis. *Urogenital System*: renal failure, azotemia, cystitis, hematuria, defective oogenesis or spermatogenesis, transient oligospermia, menstrual dysfunction, infertility, abortion, fetal defects, severe nephropathy. *Pulmonary System*: Interstitial Pneumonitis. Deaths have been reported; chronic interstitial obstructure pulmonary disease has occasionally occurred. *CNS*: headaches, drowsiness, blurred vision. Aphasia, hemiparesis, paresis and convulsions have also occurred. Leucoencephalopathy following intravenous administration to patients who have had craniospinal irradiation has been reported. CNS toxicity which may occur after intrathecal use can be classified as follows: 1) chemical arachnoiditis manifested by such symptoms as headache, back pain, nuchal rigidity, and fever; 2)paresis, usually transient, manifested by paraplegia associated with involvement with one or more spinal nerve roots; 3) leucoencephalopathy manifested by confusion, irritability, somnolence, ataxia, dementia, and occasionally major convulsions.

Other reactions related or attributed to Methotrexate, such as metabolic changes, precipitating diabetes, osteoporotic effects, abnormal tissue cell changes and even sudden death have been reported.

ANTIDOTE FOR OVERDOSAGE Leucovorin (citrovorum factor) is a potent agent for neutralizing immediate toxic effects of Methotrexate on the hematopoietic system. Where large doses or overdoses are given, Calcium Leucovorin may be administered by intravenous infusion in doses up to 75 mg within 12 hours, followed by 12 mg intramuscularly every 6 hours for 4 doses. Where average doses of Methotrexate appear to have an adverse effect, 2 to 4 ml (6 to 12 mg) of Calcium Leucovorin may be given intramuscularly every 6 hours for 4 doses. In general, where overdosage is suspected, the dose of Leucovorin should be equal to or higher than the offending dose of Methotrexate and should best be administered within the first hour. Use of Calcium Leucovorin after an hour delay is much less effective.
CAUTION: Pharmacist: Because of its potential to cause severe toxicity, Methotrexate therapy requires close supervision of the patient by the physician. Pharmacists should dispense no more than a seven (7) day supply of the drug at one time. Refill of such prescriptions should be by direct order (written or oral) of the physician only.

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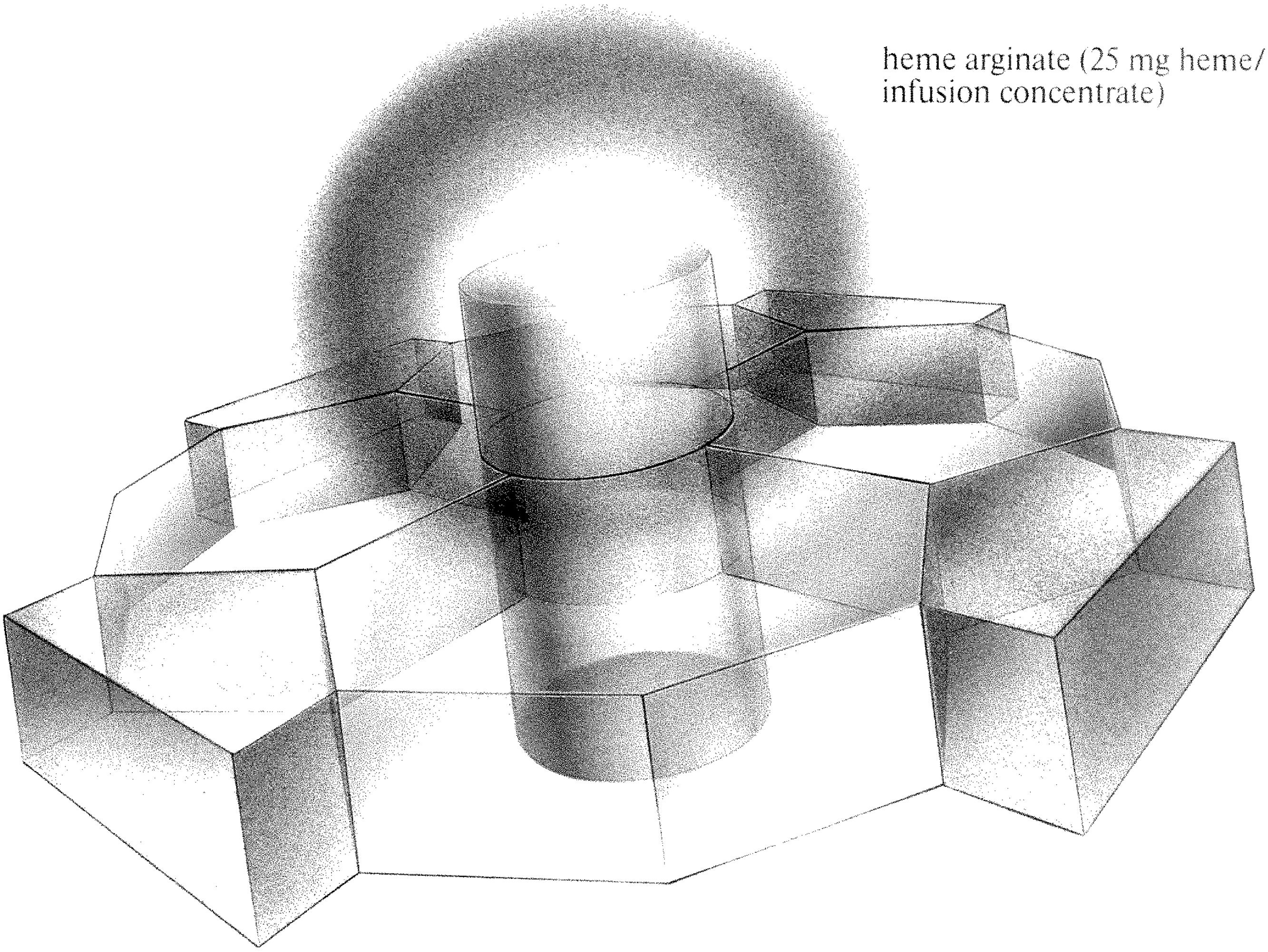

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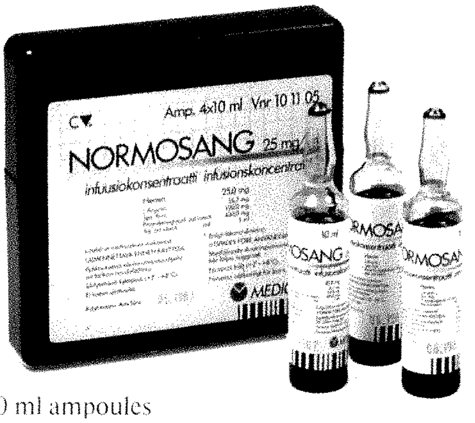
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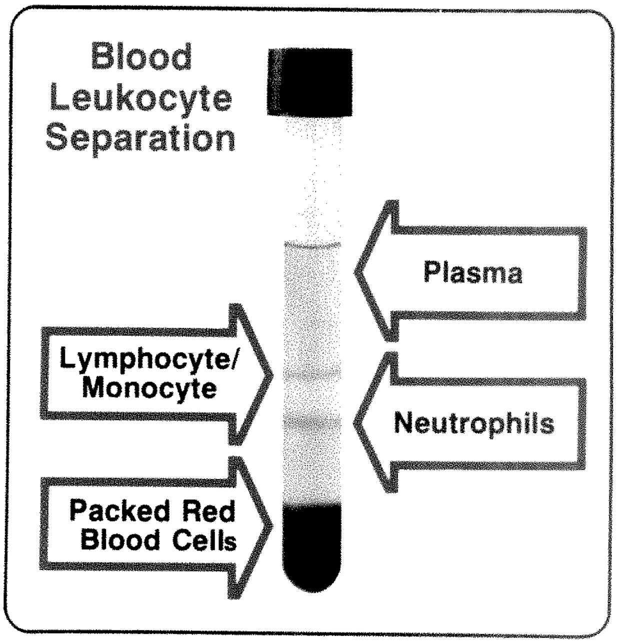

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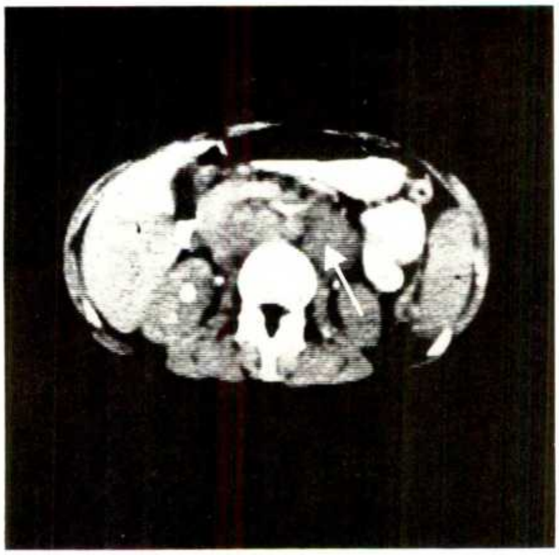

Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
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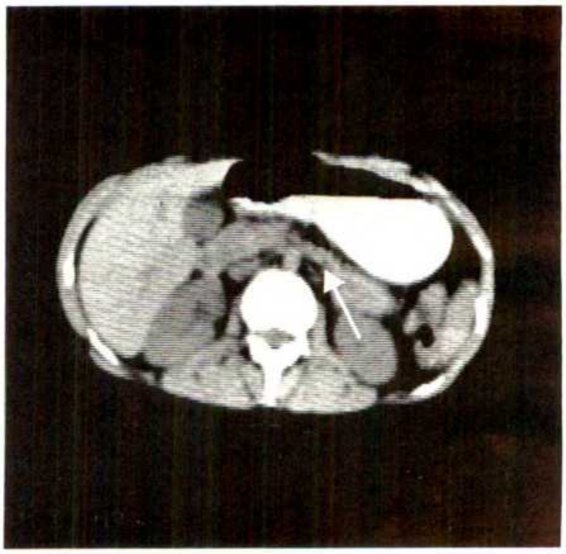

No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al:* Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al:* Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen SL *et al:* Combination chemotherapy advanced diffuse histiocytic lymphoma with the six-drug COP-BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

*Case information on file at Bristol-Myers Oncology Division

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Blenoxane® (sterile bleomycin sulfate) ...Integral Component of Chemotherapy Regimens in Non-Hodgkin's Lymphoma

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M-BACOD

4. Skarin AT, Canellos GP, Rosenthal DS, *et al:* Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
5. Anderson KC, Skarin AT, Rosenthal DS, *et al:* Combination chemotherapy for advanced non-Hodgkin's lymphomas other than diffuse histiocytic or undifferentiated histologies. *Cancer Treat Rep* 68:1343-1350, 1984.

m-BACOD

6. Skarin A, Canellos G, Rosenthal D, *et al:* Moderate dose methotrexate (m) combined with bleomycin (B), Adriamycin (A), cyclophosphamide (C), Oncovin (O) and dexamethasone (D), *m*-BACOD, in advanced diffuse histiocytic lymphoma (DHL). *Proc Am Soc Clin Oncol* 2:220, 1983.

MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

Blenoxane* *(sterile bleomycin sulfate)*

Brief Summary of Prescribing Information (1) 10 84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane* be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain $FI\,O_2$ at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban*. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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HERPES SIMPLEX INFECTION IN THE COMPROMISED HOST:

OFTEN SERIOUS, TOO OFTEN UNSUSPECTED

Number one in a series of typical case presentations.

Esophagitis in a leukemia patient

In patients with hematologic malignancies, the visceral organ most frequently involved by herpes simplex virus (HSV) infection is the esophagus.[1] Extensive ulcerative lesions may develop on the upper two thirds of the esophagus with confluent lesions of the distal third.[2] In some cases, however, the mucosa may be grossly unremarkable even when microscopic examination shows evidence of herpetic ulcerative esophagitis.[1] Similarly, some patients may display symptoms of dysphagia and burning retrosternal pain,[3] whereas others may appear asymptomatic.[1] The diagnostic method of choice for patients with suspected herpetic esophagitis is esophagoscopy with visualization of the mucosa and procurement of cytologic and biopsy specimens from ulcerated areas.[1]

References: 1. Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **2.** Wong KK, Hirsch MS: Herpes virus infections in patients with neoplastic disease. Diagnosis and therapy. *Am J Med* 1984; 76:464-478. **3.** Lightdale CJ, Wolf DJ, Marcucci RA, et al: Herpetic esophagitis in patients with cancer: Ante mortem diagnosis by brush cytology. *Cancer* 1977;39:223-226.

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Herpetic infections may result in extensive disease and death

Immunocompromised patients are highly vulnerable to HSV infection. Such infections may interrupt therapy and even lead to death if not detected early and managed aggressively.[1] Although the initial appearance may be mild, HSV infection may progress to severe localized mucocutaneous ulcerations. The greatest threat that herpes simplex poses is an infection that disseminates from a mucocutaneous or unrecognized primary lesion and involves the lungs, liver, esophagus, or central nervous system.[1] Furthermore, herpetic ulcers frequently provide an entry for secondary bacterial and fungal pathogens.[2]

Infections may not be diagnosed correctly without virologic study

Some investigators claim that the actual incidence of serious HSV infection is probably higher than reported because "the possibility of herpetic disease is insufficiently considered and appropriate diagnostic viral studies are often not carried out."[3] One study, for example, found histocytologic evidence of visceral herpes infection in 56 patients even though herpetic involvement was not suspected in any of the patients prior to autopsy.[2]

ZOVIRAX I.V. halts viral replication and speeds healing even in debilitated patients

ZOVIRAX I.V. has been shown to be a highly effective treatment for severe mucocutaneous HSV infection in immunocompromised patients. In controlled trials, ZOVIRAX I.V. rapidly halted viral shedding — within 3 days in most patients — and significantly shortened healing time as well as duration of pain.[4,5] Furthermore, ZOVIRAX I.V. may allow patients to return home sooner and enable patients with intraoral lesions to resume normal eating habits.[6]

ZOVIRAX I.V. is well tolerated by immunocompromised patients

A multicenter collaborative trial of ZOVIRAX I.V. in immunocompromised patients reported that: "Clinical benefits were obtained without appreciable toxicity. The most common reactions were irritation of peripheral veins used for drug infusion and a low incidence of rash.... Significant renal or hematologic toxicity did not occur, even in these seriously ill patients who were commonly receiving other drugs concomitantly."[5]

Note: Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes. Please see brief summary for more information.

References: 1. Feldman S, Cox F: Viral infections and hematological malignancies. *Clin Haematol* 1976;5:311-328. **2.** Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **3.** Muller SA, Herrmann EC, Winkelmann RK: Herpes simplex infections in hematologic malignancies. *Am J Med* 1972;52:102-114. **4.** Wade JC, Newton B, McLaren C, et al: Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation. *Ann Intern Med* 1982;96:265-269. **5.** Meyers JD, Wade JC, Mitchell CD, et al: Multicenter collaborative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. *Am J Med* 1982;73(1A):229-235. **6.** Data on file, Burroughs Wellcome Co.

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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously, or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: **1.** C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** J.C. Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am. J. Med. 73(1A): 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. 33:1452-1463, 1973.

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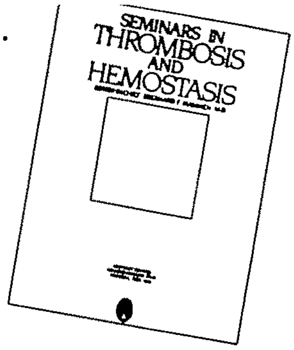

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BRIEF REVIEW

Structure, Function, and Molecular Defects of Factor IX

By Arthur R. Thompson

ACROSS 13 generations and 55 affected members, the "bleeders of Tenna" in eastern Switzerland have demonstrated that hemophilia B is not necessarily severe or associated with spontaneous bleeding episodes.[1] Moreover, patients with congenitally deficient factor IX activity are clinically indistinguishable from those with factor VIII deficiency, that is, hemophilia A. Both types of hemophilia are X-linked, and defects leading to the milder forms of either type are actually more prevalent than are clinically severe disorders.* Hemophilia B, formerly known as plasma thromboplastin component (PTC) deficiency, or Christmas disease, occurs in 1 out of every 25,000 males, accounting for nearly one fourth of all patients with hemophilia. A variety of genetic defects would be expected to explain the differences in clinical severity and clotting factor activities found in different families. Considerably more genetic heterogeneity is indicated by the variability of factor IX antigen levels among families with similar clotting factor activities.[2] Although antigen levels are often low in patients with severe hemophilia B, most patients have factor IX antigen detectable by sensitive assays using either polyclonal[2] or monoclonal antibody preparations.[3]

Factor IX is a single chain glycoprotein, representing about one ten thousandth of the total circulating protein in plasma.[4] As with other vitamin K-dependent proteins, after polypeptide synthesis there is an enzymatic modification of glutamyl residues in factor IX's amino terminal portion to form specific calcium-binding sites. In vitro, factor IX is activated by factor XIa, the "contact activation product" of the partial thromboplastin time test. Factor IX activation is the first calcium-dependent reaction in intrinsic system clotting. Unlike other steps in clotting, no protein cofactor has been identified. Furthermore, phospholipids are not required for this reaction. Factor IX can also be activated by the extrinsic system activator, factor VIIa, with its cofactor, tissue thromboplastin or tissue factor.[5] The roles of these or other activating mechanisms as related to normal blood coagulation or pathologic disorders remain to be clarified. Factor IX and, particularly, factor IXa bind specifically to the surface of endothelial cells. It remains to be established if this is the site of activation in vivo. The vascular surface may provide the site where a sufficient localized concentration is achieved to promote intrinsic factor X activation by factor IXa.[6] Phospholipid surface, such as platelet factor 3 in vivo, calcium, and a modified form of factor VIII are established cofactors in the activation of factor X by factor IXa.

Recent developments in the molecular biology of factor IX have yielded information about its genomic structure[7,8] and defined its amino acid sequence as dictated by the nucleotide coding sequence. Extensive comparisons of both genomic and protein structures can now be made with homologous regions of other proteins. Although there is as yet little evidence suggesting how the tertiary structure of the amino-terminal half of factor IX is arranged, immunochemical and binding studies should provide evidence. In contrast, the three-dimensional structure of the active site and contiguous sequences in the heavy chain of bovine factor IXa have been predicted by a computer model.[9] However, additional data are required to elucidate which portions of the factor IXa heavy chain provide substrate specificity, allow for specific cofactor binding, and interact with the amino-terminal domains of the molecule.

GENE STRUCTURE

The factor IX gene is quite large, containing nearly 34 kilobases (kb).[7,8] Only 4% of the nucleotide sequence codes for protein; this is done by eight separate exons. The gene is located near the tip of the long arm of the X chromosome,[10-12] near the fragile site defect, which is associated with a mental retardation syndrome.[13] Of homologous vitamin K-dependent proteins, there are genomic data available on prothrombin[14] and on the cDNAs for factor X[15] and protein C.[16]

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*There are currently 92 identified patients with hemophilia B from 58 families living in Washington state, a population of 4.2 million. Of these, 20% are severe (that is, having less than 1 U/dL factor IX clotting activity and the occurrence of "spontaneous" bleeding episodes), 48% are moderate (with clotting activities from 1 to 5 U/dL), and 32% are mild (with clotting activities from 6 to 26 U/dL). Note that 100 U/dL corresponds to 100% of a normal plasma pool and that the normal concentration of factor IX in plasma is about 5 $\mu g/mL$.[2]

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The genomic structure of factor IX and its relation to the gene product are represented in Fig 1. The first exon consists of a leader sequence and the signal for initiation. Translation probably begins with the codon for a Met residue which is 46 positions before the codon for the amino terminus of the mature protein.[8] The pre-pro sequence codes for a hydrophobic portion and two cysteine residues are contributed by the first exon. This is followed by hydrophilic portion coded for by part of the second exon and terminates in Lys-Arg (the last two residues before the amino-terminal Tyr of the circulating protein). The Arg-Tyr bond is then cleaved by a processing protease[8] to form the species recognized in plasma. The first and second exons are separated by an intron of about 6 kb, whereas the second and third exons have only a short intervening sequence. The balance of the second and third exons code for the Gla domain of factor IX, which contains its gamma-carboxyglutamic acid (Gla) residues. These contribute calcium-binding sites. Exons four and five are separated by a large intron, and each codes for a region in the protein that is homologous with epidermal growth factor.[17] The sixth exon is about 21 kb from the 5' end. It codes for the two activation cleavage sites with flanking sequences.

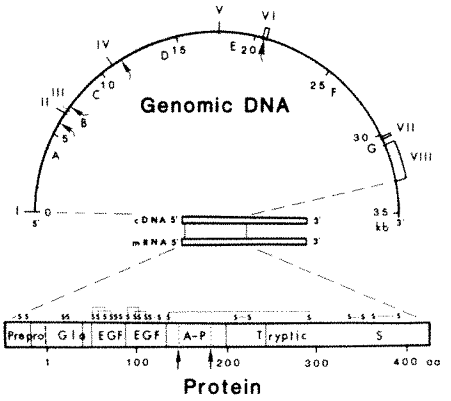

Fig 1. The genomic structure[7,8] of human factor IX is represented in the upper portion by a semicircle from the 5' (left) to the 3' (right) end. Arabic numerals refer to kilobases. Exons, numbered I through VIII, are shown on the outer sides of the semicircle, and introns, lettered from A through G, are on the inner sides of the semicircle. Positions of the four known polymorphisms are indicated by the curved arrows, including a 50-base pair insertion element polymorphic for Hinf I and Dde I near the 3' end of intron A[8,60]; a site polymorphic to Xmn I near the 5' end of intron C[60]; the Taq I polymorphism near the 5' end of intron D[8,11,56]; and the Ala-Thr polymorphism within exon VI.[61] The coding region of factor IX's cDNA is indicated in the center and below it the coding and 3'noncoding tail of factor IX's mRNA. The factor IX transcription product is indicated as an elongated rectangle at the bottom. Amino acids of the mature protein are numbered by arabic numerals. Cysteine residues are indicated by an "s," with disulfide bonds tentatively placed. Within the rectangle, solid vertical lines indicate the positions of the seven introns. "Prepro" represents the first 46 amino acids from the point of peptide synthesis initiation to the processing cleavage site; "Gla" is for the domain with gamma-carboxyglutamic acid residues; "EGF" stands for the homologous epidermal growth factor-like regions; "A-P," for the activation peptide which has Asn-linked carbohydrates and the two factor XIa activation cleavage sites (straight arrows and dotted lines); and "tryptic" for the catalytic portion, including the active center Ser, "S."

The last two exons occur at the end of the genomic sequence and are separated by a short intervening sequence. Together they code for the balance of the heavy chain of factor IXa, which shares considerable homology with tryptic serine proteases, particularly those of the clotting system.

The gene structures for prothrombin and protein C are homologous through the third exon, although with somewhat smaller introns (see ref. 8). After the third exon, the prothrombin gene has separate exons coding for two kringle-like structures; this gene is also more variable through the catalytic region, which comprises four separate exons.[13] The gene structure of protein C contains the same number and order of exons as does factor IX. It remains to be determined whether the exons for factors VII and X, and for the homologous regions of the non-protease, vitamin K-dependent proteins S and Z, are similar to those exons within the factor IX and protein C genes. Another distinctive feature of factor IX is the size of the messenger RNA. About half consists of a nontranslated 3' tail that follows the 1.4 kb which are translated.[7]

PROTEIN STRUCTURE AND FUNCTION

Human factor IX has a molecular weight (mol wt) of 54,000 and contains about 17% carbohydrate.[4] It comprises 415 amino acid residues. Their sequence, as dictated by the genomic structure, is illustrated in Fig 2. Beginning with its amino-terminal Tyr, the first 46 residues, as products of the second and third exons, make up the Gla region. Residues 47 through 127 represent the two epidermal growth factor-homologous regions, including several cysteine residues. Between amino acids 128 through 195, as coded for by exon six, are the two arginyl bonds that represent the activation cleavage sites, and the two major carbohydrate side chains. The final 220 residues are products of the last two exons and contain the active site components, the trypsin-like or catalytic portion of factor IXa. The carboxy-terminal residue, No. 415, is Thr.

Gla region. Within the first region are the 12 gamma-carboxy glutamyl residues found in factor IX, beginning with residue 7 and running through 40. These include each site within the region for which a codon specifies Glu. These strongly acidic residues were first identified by Stenflo in bovine prothrombin,[18] where only 10 are found. A vitamin K-dependent carboxylase system associated with the microsomes of hepatocytes is responsible for the posttranslational modification of Glu to Gla.[19] Within the Gla region, human and bovine factor IXs only differ at residues 11, 19, and 37. The two cysteine residues, Nos. 18 and 23, most likely form a disulfide bond, as shown for the homologous sequence in factor X and by the fact that this region of bovine factor IX can be separated without reduction and alkylation after limited proteolytic cleavage.[20]

In prothrombin, the Gla residues account for high-affinity calcium-binding sites. It is not yet known which residues are paired (or perhaps form triplets) three dimensionally. There appear to be several calcium-binding sites in human factor IX,[21,22] and at least two are high affinity.[22] Furthermore, these may be qualitatively distinct.[23] Bovine factors IX[20] and

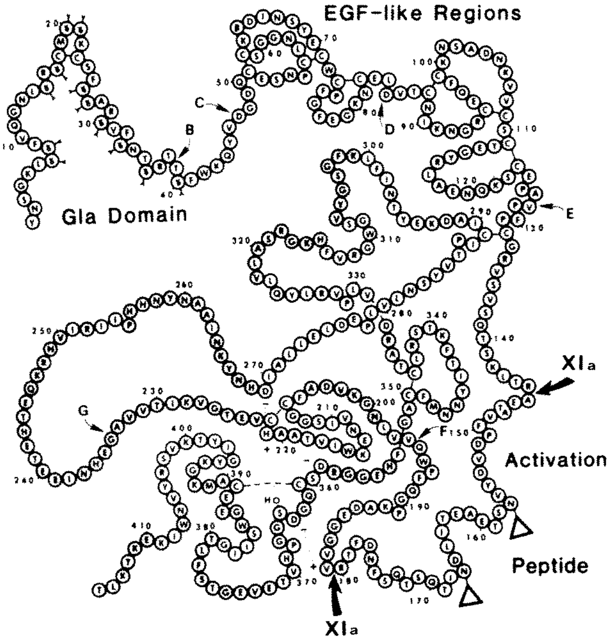

Fig 2. Amino acid sequence of human factor IX. Open circles represent amino acid residues with their standard single-letter abbreviation except that "γ" is for gamma-carboxyglutamic acid and "β" is for beta-hydroxyaspartic acid. Disulfide bonds between cysteine residues (C) are tentatively placed by homology. Residues are numbered from the amino terminal Tyr (Y) through the carboxy terminal Thr (T), residue 415. The locations of introns B through G (see Fig 1) are indicated by the smaller arrows,[8] whereas the larger arrows represent the two cleavage sites for activation by factor XIa.[4] The two Asn-linked carbohydrates in the activation peptide region are indicated by the open triangles. Regions of the heavy chain of factor IXa, which are variable in relation to homologous serine protease sequences,[9] are likely to be represented on the protein's surface and include the following residues: 199-204, 223-268, 301-307, 315-322, 354-358, and 371-380. The catalytic components include: Ser$_{365}$ (–OH), His$_{221}$ (+), and Asp$_{269}$ (–) of the active site; Asp$_{361}$ (–) of the substrate binding pocket; and Asp$_{364}$ (–), which forms an ion pair with the amino terminus of Val$_{181}$ once it has been formed by a specific activation cleavage.

X,[24] like protein C,[25] contain a high-affinity calcium-binding site once the Gla residues are removed after limited chymotryptic cleavage. This occurs at the Phe-Trp bond, residues 41 to 42, in factor IX.[20] However, it is not clear if such a site is expressed on the surface of the native, intact protein. A high-affinity site remains after chemical modification of most of the Gla residues of human factor IX as well.[26] There is indirect evidence that Gla residues function to form calcium bridges with phospholipid monolayers.[27] An alternative explanation is that this region of the vitamin K-dependent factors undergoes a marked conformational change on calcium binding and that the calcium-dependent conformer has phospholipid-binding sites exposed on its surface. This view is supported by studies with metal ion-dependent antibodies.[28] The cooperative effect of calcium binding on factor IX activation is further evidence for a conformational change.[22] If divalent metal ion-dependent conformation is confined to the Gla region, this region would somehow have to influence the activation cleavages and catalytic site of factor IX. Indeed, after chemical modification of the Gla residues, the clotting activity of factor IX is inhibited.[26] It remains possible, however, that other types of calcium-binding sites may exist elsewhere in the molecule, perhaps even in the heavy chain portion of factor IXa. The latter possibility could account for the protective effect of calcium against cleavage of factor IX by thrombin.[29,30]

Growth factor-like regions. The next two regions contain 12 of the 22 cysteine residues found in the entire factor IX molecule. Within the two regions, 12 of these 81 residues (15%) differ between the human and the bovine species. If two sets of three disulfide bonds are arranged according to homology with those of the murine epidermal growth factor, disulfide loops are formed with the bonds between the first and third, second and fourth, and fifth and sixth cysteine residues of each of the two segments.[17] Although the sizes of the two sets of loops differ (eg, residues 74 through 81 *v* 112 through 123, Fig 2), this is also seen when comparing the homologous structures of protein C, in which the first loop contains two additional cysteine residues and a considerably larger sequence.[16] An unusual amino acid, beta hydroxyaspartic acid, at the 64th position for factor IX, is also present in homologous positions in factor X and protein C.[31] It remains unclear whether or not the hydroxylation occurs by the vitamin K-dependent carboxylase system in the liver or imparts any degree of a shared calcium-binding site.[20,24]

In addition to the 10 distinct homologous regions within the murine growth factor gene,[17] similar disulfide-rich structures are present in a diverse array of proteins. These include tissue plasminogen activator where the region is coded for by a separate exon,[32] factor XII,[33] urogastrone (the human equivalent of murine epidermal growth factor), the low-density lipoprotein receptor,[34] and even the early protein of vaccinia virus.[35] Other than the ability of the growth factor hormones to bind to specific cell receptors and stimulate growth, it remains to be shown whether this region of factor IX or the other hemostatic proteins are involved in cellular interactions. It is of interest that prothrombin, being otherwise highly homologous, lacks these two structures but contains instead two kringle-like regions, each coded for by a separate exon.[14] Four kringles are present in plasminogen and include the binding site(s) for fibrin.[36] Kringle-like structures are also found in tissue plasminogen activator[32] and factor XII.[33]

Activation and activation peptide region. The next region to be discussed contains the activation peptide, including the two activation cleavage sites and flanking sequences. Near the amino-terminal end of this region is a cysteine, at position 132. By homology with chymotrypsin, a disulfide bond between this and residue 289 can be tentatively assigned, and thus account for the binding between light and heavy chains of factor IXa. The sites of cleavage by factor XIa[4] (heavy arrows, Fig 2) include an Arg-Ala bond (residues 145 to 146) and an Arg-Val bond (residues 180 to 181). It is of interest that, other than calcium, there are no known cofactors or phospholipid requirements for intrinsic system activation. Phospholipids do enhance the activity of celite-bound factor XIa somewhat.[37] Factor XI can be detected in platelets and factor XIa does bind to the platelet surface.

However, this interaction does not appear to enhance the ability of this enzyme to activate factor IX,[38] and under some conditions, a product of stimulated platelets appears to inhibit activation.[39]

Factor VIIa, with its cofactor tissue factor, also activates factor IX by cleaving the same two bonds as factor XIa in the presence of calcium.[5] From small amounts of intermediates noted on polyacrylamide gel electrophoresis, it appears that the Arg-Ala bond (residues 145 to 146) is most susceptible and is cleaved first.[4,29] A different order of bond cleavage is noted when the coagulant protease from Russell's viper venom is used. This is particularly apparent in bovine factor IX, where the Arg-Val bond (residues 180 to 181) can be cleaved almost exclusively to form factor IXa-alpha, a species with in vitro coagulant activity.[4] From the amounts of intermediate forms of factor IX seen on polyacrylamide gels,† it also appears that the human Arg-Val bond is the most susceptible to cleavage by the venom protease.[29] However, the Arg-Ala bond in human factor IX is more readily cleaved than in bovine factor IX so that the primary product is factor IXa-beta, the enzymatic form that has had both activation cleavages. Human factor IXa-alpha has been isolated and clearly has reduced procoagulant activity when compared with the beta form.[40]

Other proteases can give cleavage patterns similar to those seen on activation, even though they do not specifically cleave arginyl bonds and do not activate factor IX. These include chymotrypsin and elastase.[29,41] An interesting feature of factor IXa generation by contact activation product in vitro is that only partial activation occurs. Indeed, early, partially purified preparations of the factor IX precursor started with either human serum or plasma.[42] The mechanism(s) that inhibits or limits factor IXa formation during intrinsic system clotting of plasma is not known.

Once the two specific arginyl bonds are cleaved, the disulfide-bonded amino-terminal light chain and carboxy-terminal heavy chain migrate as a 20% smaller species than native factor IX on polyacrylamide gels. The noncovalently bound activation peptide can be demonstrated as a broad, lower-mol wt band. The activation peptide contains only 35 amino acids. With its two Asn-linked carbohydrate side chains, it has a mol wt of around 10,000 and reacts with carbohydrate stains. Bovine factor IX has a somewhat larger activation peptide due to a third carbohydrate chain on an Asn equivalent to residue 172.[4] Of interest, 13 of the 35 residues (37%) in the activation peptide differ between human and bovine species as opposed to an 85% identity of residues overall. There are no qualitative differences when activators or substrates among the two species are compared, suggesting that the peptide structure between the cleavage sites has little influence on enzymatic properties. Among other vitamin K-dependent proteases there is little homology in the peptide region; indeed, activated protein C has a peptide of only 12 amino acids without any Asn residues to bind carbohydrate.[16] Along with factor X, protein C circulates predominantly with the equivalent of the Arg-Ala, or first of two activation sites, already cleaved (and missing tri- and di-peptides, respectively), despite their cDNA's specifying single chains.[15-16]

A sensitive assay for the generation of factor IXa has exploited activation peptide formation by using ^{3}H-labeled carbohydrate residues, which become trichloroacetic acid-soluble after cleavage of both activation sites.[43,44] Although such an assay can provide precise and sensitive data for kinetic analyses, it is indirect. Especially in nonisolated systems, other proteases can cleave bonds near those responsible for activation and lead to inactivated species[29] with soluble, labeled carbohydrate peptides.

Heavy chain of factor IXa. As in the three-dimensional structures of trypsin and chymotrypsin, the generation of a free amino group of the hydrophobic Val residue 181 in factor IX, allows ion pair formation with the free carboxyl group of Asp at position 364.[4,9] This would account for activation of the active center Ser, at position 365, by making its hydroxyl group more labile. Catalysis of a peptide bond is facilitated by His at 221 (within a typical disulfide loop toward the amino terminus of the heavy chain) and Asp at 269, which are essential components for the active site. The positively charged side chain of a specific substrate's Arg residue binds ionically in the substrate binding pocket of factor IX, which has its negative charge imparted by the Asp at position 359. Secondary sites for substrate binding to trypsin-like proteases include the Ser at residue 384 and two Glys at positions 386 and 395 in factor IX. The disulfide bond shown between residues 361 and 389 is likewise a common feature.

Factor IXa, a highly restrictive protease, is relatively slow to react with low-mol-wt substrates and inhibitors. Indeed, attempts to find specific substrates with peptide sequences including the bond cleaved in factor X have been unsuccessful.[45] This may in part relate to its need for interaction with a modified form of factor VIII, in addition to phospholipids and calcium, before expressing potent coagulant activity. Thus most assays for factor IXa activity have required the addition of a modified factor VIII and measured, as an end point, the coupled activation or cleavage of factor X.[5,46,47] To probe the active site of factor IXa more directly, a labeled, nonspecific protein inhibitor, antithrombin III, has been used.[29] In the presence of heparin, this forms a stable or covalent-like intermediate with the active form of most coagulant serine proteases, including factor IXa.[48] Specificity was then provided by an insolubilized monoclonal antibody to human factor IX.[3] It was found that factor IX in a complex with the antibody could be activated and bind the labeled antithrombin III to its active site, such that the antibody did not interfere with inhibitor binding. With the labeled inhibitor immunospecific assay, it was possible to show that the activation-like cleavages of factor IX by chymotrypsin and elastase were not associated with generation of an active site; trypsin both partially activated and inactivated factor IX.[29]

It appears that factor IXa can slowly activate factor X,[49] and the basal rate is increased somewhat by phospholipid and calcium.[46,49] With an unmodified form of factor VIII, a lag phase can be seen before generating factor Xa converting activity; the lag is abolished by addition of thrombin-modified factor VIII.[47,50] The latter markedly enhances the activation cleavage of factor X by factor IXa. There is indirect evidence that modified factor VIII interacts with the heavy chain of factor IXa. This evidence is provided by experiments with a monoclonal antibody to factor IX that reacts with the heavy chain portion of reduced factor IXa and interferes with acceleration of factor X cleavage by the modified cofactor.[51] An additional feature of this interaction is that factor IXa markedly stabilizes the activity of thrombin-modified factor VIII to proteolysis by activated protein C.[50]

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†An inactive intermediate species, consisting of the activation peptide heavy chain (residues 146 through 415) can be seen during activation by factor XIa or factor VIIa tissue factor. A smaller intermediate, corresponding to the light chain activation peptide (residues 1 through 180) is present when activation has been by snake venom protease; the second intermediate is referred to as factor IXa-alpha because it has enzymatic activity.[4,29,40]

In comparing the three-dimensional structures of chymotrypsin and trypsin with the primary sequence data from thrombin and the heavy chains of bovine factors IXa and Xa, Furie et al[9] designated six areas of the heavy chain in which the structure was variable (see Fig 2). In general, these portions of the factor IX molecule can be predicted to be exposed on its surface and correlate with major differences in considering subsequent data on the human and other homologous proteins. For example, the second and largest variable region occurs after the active site His and extends up to the active site Asp (residues 223 through 268). This large loop differs in ten residues in comparing bovine and human species; the former also has an Asn-linked carbohydrate side chain attached to residue 260. From the prevalence of charged residues, especially in the sequence from residues 235 through 249, one would predict strong antigenicity, as shown on the surfaces of other proteins. Considering the heavy chain, complete identity in human and bovine species is found from Asp 359 to the carboxy terminus. The carboxy-terminal tail, from residue 409 through 415, shows less homology among the different hemostatic proteins and may well constitute an additional (seventh) variable region. In factor IX, it contains three positively charged and one negatively charged residues. In contrast, human factor X has an additional 19 amino acids and its bovine counterpart has 24 more residues, including non–Asn-linked carbohydrate.[15,47]

GENETIC POLYMORPHISMS AND SCREENING

Analysis of the gene product for either heterozygote detection or prenatal diagnosis is problematic. Nearly half the women who are obligate heterozygotes for hemophilia B have levels of factor IX clotting activity in their plasmas that are beyond two standard deviations below the normal mean.[52] Determination of factor IX antigen levels can improve precision somewhat over results from clotting activity assays alone, and in some women, circulating abnormal antigen may be detectable to aid in the diagnosis of the heterozygous state.[2] Occasionally, families may be found whose abnormal gene product reacts to polyclonal antibodies but not to a specific monoclonal antibody.[3] Heterozygote detection, however, is confounded by the wide, normal distribution of factor IX levels and random X chromosome inactivation. It is impossible to exclude the carrier state when a woman's factor IX level (ie, the gene product) is within the normal range without additional data from gene analysis.

Prenatal diagnosis has been accomplished in a few cases with an immunoassay based on a specific alloantibody. The first family studied had severe hemophila B, but the mother also had low antigen levels.[53] Variables to consider during mid-gestation, however, are the significant amounts of factor IX in normal amniotic fluid and the low levels in normal fetal plasmas.[54] Due to the difficulties with gene product analysis and the presence of a wide variety of genetic defects, it is more attractive to identify polymorphisms within the factor IX gene and determine how they segregate within specific families. Linkage of a polymorphism to the hemophilic gene can provide a direct approach for both heterozygote detection and prenatal diagnosis.

The first recognized polymorphism within the factor IX gene was the presence of an additional Taq I restriction endonuclease cleavage site occurring just 3' to the fourth exon.[11,55] About 40% of women are heterozygous for this intragenic polymorphism, making it an ideal marker.[55-59] Two additional frequent restriction site polymorphisms have been identified (see Fig 1) such that 65% to 70% of women are heterozygous for one or more of these intragenic sites.[60] By sequencing individual cDNAs, and amino-terminal amino acid sequencing of factor IXa's (including the activation peptide) from different individuals, an additional polymorphic site has been defined.[61] This site corresponds to the codon for residue 148, which is near the amino terminus of the activation peptide. A dimorphism had been suggested by disparate results of the corresponding codon from different genomic libraries. The population frequencies of the polymorhpic residues are not yet available, but the Thr allele appears to be more prevalent.[61] There is no known endonuclease that recognizes this difference, but synthetic oligonucleotide probes could be designed.

At least one polymorphism appears to be present within the heavy chain region of factor IXa. A monoclonal antibody reacting with the heavy chain shows decreased reactivity in plasmas for a significant portion of males, when compared with antigen levels using polyclonal or different monoclonal antibodies.[62] Polymorphism to another monoclonal antibody preparation has also been described.[63] Analysis of polymorphisms of the gene product may, in some cases, be helpful in assessing linkage for detection of heterozygotes. Where applicable, they could avoid the more extensive approach of DNA analysis. It is conceivable that monoclonal antibodies could be made to conjugated synthetic peptides containing, for example, sequences including the respective polymorphic amino acids at residue 148. If these reagents react with native factor IX, they could be used to screen for the polymorphism in the protein.

HEMOPHILIC DEFECTS

Immunologic data suggest that there are a wide variety of genetic defects which account for hemophilia B in different families.[2,3,58,64] Elucidation of the structural bases for these defects in comparison to the functional alterations they incur should provide important data on the mechanisms of activation and action of factors IX and IXa. Data from the several hemophilic defects analyzed to date will be summarized. An additional review of this subject has been written.[65]

Using subgenomic and cDNA probes to factor IX, Giannelli and co-workers looked at the DNA of patients with hemophilia B and circulating inhibitors.[66] Four of the five patients with inhibitors showed gross deletions within their factor IX genomic structures. Curiously, two of them were related but were deleted at different sites. An additional case with an inhibitor was reported to have a partial gene deletion.[67] Another family showed deletion of a 10-kb segment containing the fifth and sixth exons of factor IX, as described by Chen et al.[59] Seven affected members of this family have severe hemophilia B without inhibitors. Therefore, a deletion per se does not mean an inhibitor will occur. Small amounts of a partial gene product with a mol wt of approximately 36,000 were detected in the urines of these patients and of obligate heterozygotes from this family.[68] Antibodies specific for the calcium- dependent conformation of factor IX (ie, its amino terminal end) were also helpful in demonstrating trace levels of antigen in the patients' plasmas. In addition to their interests as a type of genetic defect, partial deletions can serve as markers for heterozygote detection or prenatal diagnosis within the families in which they are found.[59,68]

There have been two hemophilic factor IXs in which an amino acid substitution was identified in unrelated patients with mild to moderately severe hemophilia B. These patients had normal levels of circulating factor IX antigen. The first, factor $IX_{Chapel\ Hill}$, was identified by amino acid sequencing of a tryptic peptide.[69] It was known that this abnormal protein showed a different activation cleavage pattern when electrophoresed on gels, suggesting that only the Arg-Val and not the Arg-Ala activation cleavage site could be hydrolyzed. This was due to an Arg-to-His substitution at residue 145 such that this patient could only form a mutant enzyme that is equivalent to factor IXa-alpha.[40] The second defect has a substitution of Gly for the native Asp at position 47, as determined by sequencing of the cDNA in a patient with factor $IX_{Alabama}$.[70] It is not yet clear how the loss of this negatively charged residue affects the function of factor IX, as there appears to be no difference in calcium binding. Loss of a charged residue does support the concept of a cooperative effect between the light chain and catalytic portions of factor IX. Although earlier studies suggested abnormal binding of factor $IX_{Alabama}$ to phospholipids, on more detailed analysis, binding to lipid vesicles was normal in the presence of calcium.[71] The nucleotide substitution, occurring in a codon that spans the third and fourth exons, could represent an uncommon polymorphism found coincidentally, but it probably accounts for the patient's hemophilia.

Of other abnormal factor IX proteins that have been partially characterized, factor $IX_{Zutphen}$ has an extra peptide that is disulfide bonded.[72] On reduction, the abnormal factor IX is of normal size. The defect is presumably associated with the amino terminal portion of factor IX, where it interferes with the normal calcium-dependent properties in the Gla region of this abnormal protein. It is possible that either of the cysteine residues, at position 18 or 23, has been substituted. This substitution would leave a free sulfhydryl group to bind to another peptide, perhaps even the leader sequence (which contains cysteines at the 19th and 29th residues before the amino terminus). An abnormal factor IX antigen with similarly altered calcium-binding properties appears to result from substitution at the processing protease cleavage site, producing a somewhat larger, albeit single-chained, protein.[73] This defect is referred to as factor $IX_{Cambridge}$.

Several other factor IX proteins have been shown to have either normal or somewhat altered activation cleavage rates.[74,75] Hemophilia B_m is an uncommon type in which there is an associated prolongation of the prothrombin time when ox brain, but not human brain, is used as the source of the thromboplastin.[76] The basis for how the circulating, abnormal factor IX inhibits the extrinsic activation of factor X by factor VIIa when bovine thromboplastin is used remains to be determined.[75,77,78] There appears to be some heterogeneity among different families with the B_m defect, but all patients have had normal levels of circulating factor IX antigen. The molecular defect in a final, isolated factor IX disorder, referred to as hemophilia B Leyden,[79] will be of considerable interest, as it may relate to factors that control the circulating levels of this protein. Only two families have been described and the factor IX levels improve with increasing age, especially after puberty. Congenitally low levels of all four vitamin K-dependent clotting factors have been described.[80-82] These cases are best attributed to abnormalities within the vitamin K-dependent carboxylase and epoxide reductase systems, and the genes for the clotting factors are likely to be normal.

It is anticipated that the list of defined structural defects will proliferate rapidly, given the currently available data on the genomic and amino acid sequences of factor IX. Relation of the molecular defects in the various types of hemophilia B to the specific functional abnormalities they cause will further our knowledge of the role of this protein in the normal mechanisms of blood coagulation. Means to evaluate the potential for its activated form to lead to thrombosis or disseminated intravascular coagulation may then emerge.

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Production of B Cell Growth Factor(s) by Neoplastic B Cells From Hairy Cell Leukemia Patients

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Recent studies have shown that normal human T cells contain a high-molecular-weight (mol wt) protein exhibiting B cell growth factor (BCGF) activity. Other studies have shown that virally transformed human B cells also secrete a high-mol-wt BCGF-like molecule in vitro. We have studied neoplastic B cells from patients with untreated hairy cell leukemia (HCL) to ascertain whether such cytoplasmic BCGF activity is present in the tumor cells. Studies on HCL cells from four patients indicated that BCGF-like activity was in fact present in the cytosolic extracts when tested on autochthonous HCL cells as well as on normal BCGF-dependent human B cell lines. Chromatographic analysis indicated that the BCGF activity from HCL cells was similar in mol wt as well as function to the normal T cell-derived cytosolic BCGF activity. These studies suggest that HCL cells contain and, in some cases, secrete a high-mol-wt growth factor that can be autostimulatory and appears to resemble a similar growth factor molecule found in normal human T cells.

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NORMAL HUMAN B CELL GROWTH has been shown to be dependent on cellular activation by antigen or mitogen,[1,2] followed by the stimulatory action of the T cell lymphokine, B cell growth factor (BCGF).[3,4] Human BCGF has been reported to be heterogeneous with both low (14 to 16 kd)[5,6] and high (50 kd)[7] molecular weight (mol wt) forms described. Recently, a high-mol-wt form (>50 kd) of BCGF has been described in the cytosol of normal human T cells that may represent a precursor molecule for the low-mol-wt secreted form of BCGF.[8] Neoplastic human B cell growth, however, has long been an enigma, since the usual requirements for growth stimulation appear to have been circumvented by apparently autonomous cell growth. We have recently shown, however, that a variety of human non-Hodgkin's lymphomas (NHL) can proliferate in vitro in response to greatly purified BCGF,[9] indicating that these neoplastic B cells have retained the functional capacity to respond to the homologous normal growth factor. We report here that hairy cell leukemia (HCL) cells, a novel type of human B cell neoplasm that expresses the Tac antigen and rearranges Ig genes,[10] contain a high-mol-wt BCGF molecule in the cytoplasm and, in at least some cases, secrete a molecule(s) with BCGF activity. This HCL-associated growth factor can stimulate in vitro cell growth in both autochthonous HCL cells and growth factor (BCGF)-dependent normal human B cell lines.

MATERIALS AND METHODS

Patients. Newly diagnosed untreated patients with HCL were studied at the time of initial presentation. Diagnosis was usually made by bone marrow needle biopsy and aspiration using conventional histopathologic criteria,[11] including cytochemical staining for tartrate-resistant acid phosphatase (TRAP).[12] Four patients with the leukemic form of the disease (WBC > 20,000 μL) with high numbers of HCL cells in the peripheral blood were either leukapheresed or phlebotomized to obtain large numbers of neoplastic B cells.

Cell preparation. Peripheral blood collections from either leukapheresis or venipuncture were separated into peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque (FH) density gradient centrifugation. The PBMCs were washed and rosetted using neuraminidase-treated sheep red blood cells (SRBC; E_n) overnight at 4 °C. The rosetted PBMC population was then separated into SRBC rosette positive (E_n^+) or negative (E_n^-) populations on FH gradients. E_n^- cells were then placed onto plastic Petri dishes to remove adherent cells. The nonadherent E_n^- cell population was then washed and prepared for immunophenotyping.

HCL phenotyping. The E_n^- cell population was examined by immunofluorescence with a standard battery of monoclonal antibodies (MAb) for human T and B lymphocytes. The MAb included pan T cell (OKT11, T3; Ortho, Raritan, NJ); pan B cell, B1,[13] B4 (Dr L. Nadler, Dana Farber Cancer Center, Boston); myeloid-monocyte (OKM1; Ortho); and the HCL-associated MAb HC1 and HC2[14] (Dr D. Posnett, Rockefeller University, New York). HCL cases were also studied with the combination of the MAb Leu-14 (pan B) and Leu-M5 (monocyte subset) (kindly provided by Dr N. Warner, Becton Dickinson, Mountain View, Calif), which, when expressed together, can discriminate HCL cells from normal B cells present in a leukemic cell population.[15] The anti-Tac MAb[10] was a gift from Dr T. Waldmann (National Institutes of Health, Bethesda, Md).

In vitro growth factor production. Fresh HCL cells obtained from FH gradients were enriched for B cells by double E_n rosetting, followed by adherence to plastic Petri dishes. The resulting cell populations were ≥90% HCL B cells by immunophenotyping and were ≥90% viable by trypan blue staining. The HCL B cells were then set up in vitro in RPMI 1640 (Irvine, Irvine, Calif) at 2×10^6 cells per milliliter in 1% fetal calf serum (FCS; Hyclone, Logan, Utah) or 1% bovine serum albumin (BSA; Sigma Chemical Co, St Louis) and incubated at 37 °C for 24 hours in 5% CO_2 atmosphere. The culture supernatants were collected by pelleting the cells in a refrigerated centrifuge at 800 g for ten minutes and were tested for BCGF activity on growth factor-dependent human B cell lines[16] or on autochthonous HCL B cells.

Cytosolic BCGF activity from HCL cells. Cytoplasmic extracts from neoplastic HCL B cells were prepared and assayed for by the methods described by Sahasrabuddhe et al[8] for normal human T lymphocytes. Briefly, the tumor cells were pelleted and resuspended in hypotonic buffer containing 20 mmol/L HEPES (pH 7.5), 1.4 mmol/L Mg $(AC)_2$, 3.6 mmol/L $CaCl_2$, and 2 mmol/L 2-mercaptoethanol. The tumor cells were lysed with 40 strokes in a glass homogenizer and the lysate was centrifuged at 1,500 g for ten minutes to remove nuclei. The supernatant was centrifuged at 10,000 g for ten minutes to remove mitochondria and then ultracentrifuged at 100,000 g for 90 minutes to remove cell membranes and

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to obtain soluble cytosolic proteins in the supernatant. The cytosolic extract was concentrated by lyophilization and either assayed for BCGF activity or fractionated on a Sephacryl 200 (Pharmacia Fine Chemicals, Piscataway, NJ) gel filtration column.

Microassays for in vitro growth factor activity. Growth factor preparations from neoplastic human HCL B cells were assayed either on continuous BCGF-dependent human B cell lines[16] or on the autochthonous tumor cells from which the growth factor was derived. The normal B cell lines were maintained in log phase growth in the presence of RPMI 1640 medium, 3% (vol/vol) heat-inactivated FCS, and greatly purified BCGF preparations.[3] For the assay, B cells (≥95% sIg, B1$^+$) cultured for 72 hours were washed extensively in medium (four to five times) to remove any residual bound growth factor. The assays were performed in triplicate in flat-bottom 96-microwell plates (Corning Glass, Corning, NY), using a total of 1.0 to 1.5 × 10^4 cells per well in a final volume of 200 μL. The assays in which the target cells were exposed to serial dilutions of growth factor preparations were performed in RPMI 1640 supplemented with 2% (vol/vol) FCS for 48 hours. Sixteen hours before harvest the cells were labeled with 0.5 μCi (6 Ci/mmol) of ^{3}H-thymidine (^{3}H-Tdr; New England Nuclear, Boston) and harvested, using an automated micro cell harvester (Brandel, Palo Alto, Calif). ^{3}H-Tdr incorporation was determined using a liquid scintillation spectrometer (Beckman LS-8100, Palo Alto, Calif).

Tumor cell growth factor activity was assayed on freshly prepared or cryopreserved immunophenotyped HCL B cells after E_n rosetting and adherent cell removal. The HCL cells were plated at an initial cell density of 1 × 10^5 cells per well. The tumor growth factor preparations were then titrated into the wells at a final volume of 0.2 mL per well. The microwell plates were incubated for 72 hours at 37 °C in a 5% CO_2 atmosphere. The cells were then labeled with ^{3}H-Tdr for the last 16 hours of incubation period and harvested as described.

Gel filtration chromatography. Tumor cell supernatants and cytosolic extracts from HCL cells were chromatographed on a Sephacryl 200 column (2 × 100 cm) that had been calibrated with a variety of mol wt protein markers. The column was eluted in phosphate-buffered saline (PBS), 10 mmol/L phosphate, pH 7.0, with 5-mL aliquots collected per fraction. The proliferative activity of the eluted samples was then tested on growth factor-dependent cell lines in 72-hour ^{3}H-Tdr incorporation assays as described.

RESULTS

Characterization of HCL cells. Four patients with the clinical and pathologic diagnosis of HCL were studied. All had the leukemic form of the disease (>20,000 WBC/μL; ≥80% leukemic cells by morphology), and the cells studied were collected either by leukapheresis or by venipuncture. The tumor cells were enriched for by double E_n rosetting, followed by plastic adherence. The E_n negative cell populations obtained appeared to contain only HCL cells by morphological criteria and were ≥90% TRAP positive. Table 1 shows the phenotypic characterization of the HCL cells, which demonstrate that the tumor cells had standard B cell membrane markers, including cell surface immunoglobulin (sIg) and the characteristic pan B cell antigen recognized by the MAb B1. In addition, however, the myeloid-monocytic MAb, OKM1, and Leu-M5 were also present on most, if not all, of the neoplastic cells. The HCL cell population also contained the Tac antigen, which is associated with the receptor to human T cell growth factor but has also been found on some normal human B cells. The combination of the pan B cell MAb, Leu-14 labeled with fluorescein (FITC), and Leu-M5 labeled with phycoerythrin (PE) stained HCL cells simultaneously, while single-stained cells identified occasional residual normal B cells or monocytes, respectively. Further analysis by sIg light chain typing for assessing monoclonality of B cells and nonspecific esterase for monocytes indicated that ≤5% of the total HCL cell population represented these contaminating cell populations.

Table 1. Cell Surface Phenotype of HCL Patients

HCL Patient	sIg*	κ/λ	B1	Leu-14/M5†	HC2	Tac	OKM1	T_{11}
1	94	93/2	86	91	87	73	84	3
2	96	98/1	92	93	90	81	86	2
3	88	80/3	93	90	90	85	81	4
4	89	90/2	83	78	88	75	80	3

PBMCs from untreated HCL patients were separated on FH gradients and then rosetted with neuraminidase-treated SRBC (E_n) overnight at 4 °C. The E_n negative cells were then depleted of adherent cells and immunophenotyped for cell surface antigens.

*E_n-HCL cells were assayed using mouse monoclonal antibodies by indirect immunofluorescence with appropriate controls. The number shown represents the percentage of positive cells staining with each of the designated reagents.

†Leu-14, Leu-M5 double-staining percentage with FITC and phycoerythrin conjugated reagents, respectively.

Production of growth factor activity by HCL cells. HCL cell populations were set up for in vitro culture in either low-serum (1%) or serum-free (1% BSA) conditions and incubated for 24 hours at 37 °C in a 5% CO_2 atmosphere. At the termination of the culture period, the cells were centrifuged and the culture supernatants were removed. Cell viability at this time was ≥80%. The supernatants were then either frozen for future studies or used immediately in in vitro proliferative assays using either long-term growth factor-dependent human B cell lines or autochthonous HCL cells that had been maintained in vitro for 24 to 36 hours. Table 2 shows a representative example of these studies, where it can be seen that the populations of HCL B cells

Table 2. HCL Culture Supernatant Autostimulatory Activity

Target Cells	Bkgd	SN (1% BSA)			
		5%	10%	20%	30%
HCL Patient 1	132	990	1263	1725	2752
HCL Patient 2	156	680	1560	2580	ND
BD23	480	981	1867	4332	10175
BDk9	320	684	1424	4427	9564

HCL supernatants (SN) were generated at a final density of 2 × 10^6 cells per milliliter from E rosette and adherent cell-depleted tumor cell populations that were cultured for 24 hours in RPMI 1640 + 1% BSA. Two SN from each patient were obtained and tested. HCL cells are the B cells isolated from the patient's peripheral blood lymphocytes by negative selection procedure described in Materials and Methods. BD23 and BDk9 are BCGF-dependent normal B cell lines. HCL cells (1 × 10^5, >95% viable) or normal B cells (1.5 × 10^4, BD23 or BDk9 cell line) were cultured in microwells for 48 hours with varying dilutions of HCL-derived supernatants. Microwell cultures were labeled with 1 μCi of ^{3}H-Tdr for the final 24 hours of the 72-hour culture period. Results are expressed as mean cpm of triplicate assays. Standard errors were less than 10%.

release a soluble factor into culture supernatants that stimulate proliferation in both long-term human B cell lines as well as autochthonous, freshly obtained HCL cells in vitro. The release of the growth factor appears to be spontaneous in all patients studied, although the amount of activity varies somewhat from patient to patient. Growth factor activity does not seem to require lectin stimulation. In fact, lectin (PHA, protein A) stimulation does not augment the release of the growth factor. Also, it was found that the BCGF cytoplasmic activity from normal T cells would also stimulate ^{3}H-Tdr incorporation in the HCL cells (data not shown).

High-mol-wt growth factor activity in the cytosol of HCL cells. Normal human T lymphocytes have been shown to contain a high-mol-wt (>60 kd) BCGF activity in their cytosol, which may be a precursor molecule for the low-mol-wt (14 to 16 kd) secreted form of human BCGF. We were interested to ascertain whether neoplastic human B cells, such as HCL, contained such a molecule. For these studies, we purified populations of HCL cells as before and prepared cytoplasmic extract to obtain a cytosolic protein fraction. The cytosolic fraction was assayed on long-term human B cell lines as before to determine whether BCGF activity was present. Table 3 shows examples of the cytosolic fraction from two patients' HCL B cells that were positive when tested for BCGF activity on long-term BCGF-dependent cell lines. This BCGF activity was also found to titrate in a dose–response manner similar to the cytosolic BCGF obtained from normal human PBMCs. The cytoplasmic BCGF activity from normal PBMCs has been previously shown to be exclusively present in T lymphocytes.[8]

Chromatographic characterization of cytosolic BCGF activity from HCL cells. Because we had identified BCGF activity in the cytosolic compartment of HCL B cells, it was of interest to compare this factor with the cytosolic fractions obtained from lectin-activated normal human PBMCs. Therefore, the concentrated cytosolic extract from HCL B cells was chromatographed on a Sephacryl 200 column that had been calibrated with a variety of standard protein mol wt markers. Figure 1 shows the elution profile of the cytosolic and secreted BCGF-like activity from HCL B cells as compared with the similar cytosolic activity obtained from normal human T lymphocytes. It can be observed that the BCGF activities from both sources eluted from the column in a similar manner, suggesting that the growth factor activities obtained may share biochemical as well as functional similarities.

Table 3. Cytoplasmic BCGF Activity From HCL Cells

Target Cells	Cytoplasmic Growth Factor Added to Cultures (% vol/vol)	Source of Cytoplasmic Growth Factor ^{3}H-Tdr Incorporation (cpm)		
		Normal PBMCs	HCL Patient 1	HCL Patient 2
BD23 B cells	0*	198	166	198
	0.2	3738†	3740	1620
	0.6	7796	4720	2640
	1.2	9489	3936	1322
	2.5	8962	5396	3633
	3.0	ND	6794	4890
	10.0	ND	8512	ND
	20.0	ND	9486	ND

Target cells for evaluating cytoplasmic BCGF activity consisted of long-term BCGF-dependent B cell lines used at 13,000 cells per well.

*Cytoplasmic growth factor activity obtained from fractionated E_n-negative HCL that were >90% TRAP, sIg, κ, B1, Leu-14/M5, Tac positive.

†Mean cpm of triplicate assays of 72-hour cultures, labeled for final 24 hours with 1 μCi ^{3}H-Tdr. Standard errors were less than 10%.

DISCUSSION

The control of cell growth in malignant human B cells is an area of considerable interest and importance to the immunobiology of these neoplasms as well as to their clinical behavior. The description and characterization of human B cell growth factor for normal B cells[17] has greatly increased our understanding of how normal B cell proliferation is

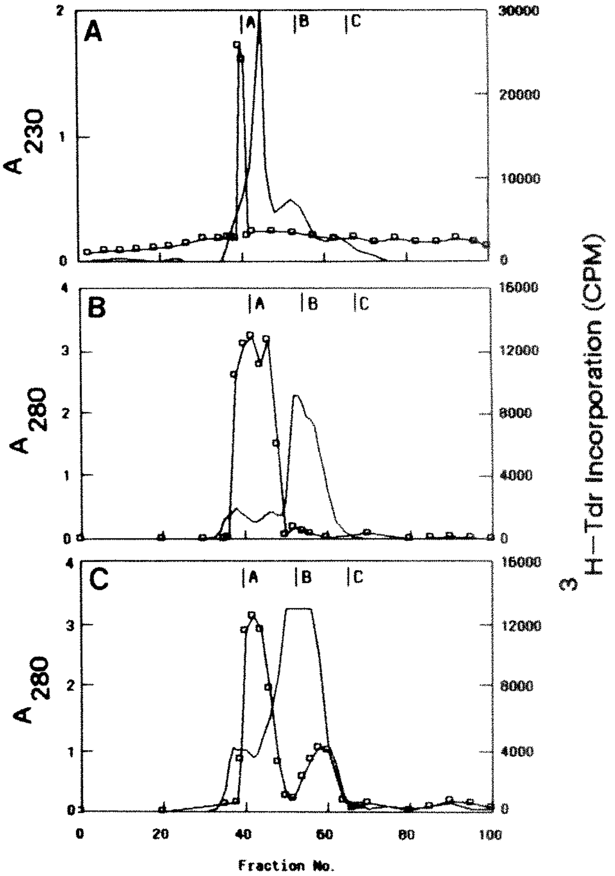

Fig 1. Composite figure showing Sephacryl 200 chromatograms of HCL cell culture supernatants (C), cytosol from purified HCL cells (B) as compared with the cytosolic BCGF activity obtained from normal human PBMCs (A). Proliferative activity (^{3}H-Tdr incorporation) was assayed on growth factor-dependent human B cell lines (□) at a final concentration of 10% (vol/vol). Absorbance at A_{230} or A_{280} is shown in the solid lines without symbols. Mol wt markers (Bio-Rad, Richmond, Calif) used for calibrating the gel column were (a) gamma globulin (158 kd), (b) ovalbumin (44 kd), and (c) myoglobin (17 kd).

regulated through the secretion of soluble factors.[18,19] Our recent studies have shown that neoplastic human B cells from a variety of non-Hodgkin's lymphomas and chronic leukemias can be stimulated to proliferate in vitro with BCGF.[9] These studies suggest that normal homologous (ie, B cell lineage) growth factor(s) can at least stimulate neoplastic B cell growth in vitro experimentally. The secretion of BCGF under the usual immunologic circumstances appears to be a tightly regulated control mechanism, similar to interleukin 2 regulation that is mediated through various types of accessory cell interactions and their soluble cytokine products.[20,21] It would therefore seem unlikely that the reactive (normal) accessory cells usually present either in neoplastic lymphoid lesions or in the peripheral blood in the chronic B cell leukemic state could account for the necessary BCGF activity, which is presumably needed to drive the neoplastic proliferative process. A likely source for such growth factor activity might then be the neoplastic B cells themselves. Since ample experimental precedence for autocrine growth factor mechanism(s) in tumor cells exists[22,23] and recent studies have indicated that Epstein-Barr virus-transformed human B cells can secrete a BCGF-like factor,[24-26] the prediction would be that at least some type of growth factor activity should be present in the culture supernatants if the tumor cells can in fact secrete the factor. Similarly, such a factor(s) could be found in the cytoplasm of the tumor cell itself, as is the case in the normal T lymphocyte.

The important recent observation that a high-mol-wt BCGF molecule is present in the cytoplasm of normal T cells, possibly representing a precursor molecule for the low-mol-wt secreted form of BCGF,[8] suggested the possibility that a similar type of system might occur in neoplastic B cells. We have subsequently observed that both secreted and cytoplasmic factors with BCGF activity could be demonstrated in fresh HCL cells. The cytoplasmic BCGF from the HCL cells appears to be similar, if not identical, to that found in normal human T cells, but the HCL BCGF activity does not show as sharp a peak after elution from Sephacryl 200 gel filtration chromatography. Also, the elution profile appears to indicate that the mol wt of both the normal PBMCs as well as the HCL cytoplasmic growth factor activity is greater than 100,000 when compared with the elution of the mol wt markers used to calibrate the column. However, recent studies indicate that when the normal cytoplasmic BCGF is purified to homogeneity, it has a mol wt of approximately 60,000, as determined more accurately by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[27] The elution profiles shown in Fig 1 compare the normal and HCL cytoplasmic growth factors, but this technique gives only the general range of mol wt. When taking these considerations into account, we believe that the actual mol wt of the HCL cytoplasmic growth factor will correspond to that found in normal PBMCs.

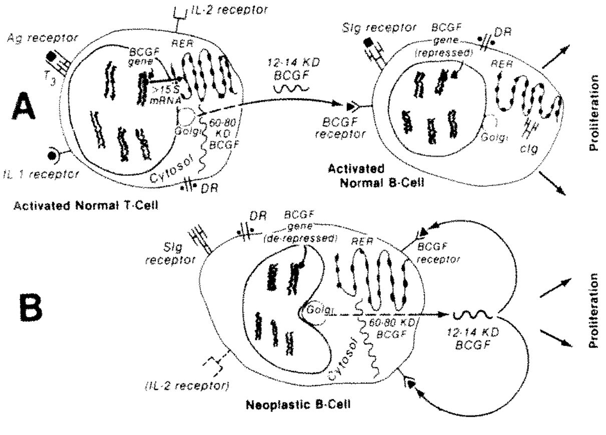

Fig 2. Comparison of a hypothetical scheme for the control of neoplastic B (HCL) cell growth (B) with a schematic representation of current concepts involved in the control of proliferation of normal human B cells (A). Neoplastic B cell growth is hypothesized to result from the expression of the gene for BCGF, which is not usually expressed in normal B cells, resulting in an autocrine loop mechanism where the neoplastic B cell can produce its own growth factor. The tumor cell, which constitutively expresses the receptor for the growth factor, then can respond to the autochthonous factor by proliferating.

These findings suggest an autocrine model of neoplastic B cell proliferation exemplified by HCL, which is shown in Fig 2, and contrasted to a current conceptual model for the control of normal B cell proliferation.[8] In this model, neoplastic B cells (HCL) contain a high-mol-wt BCGF molecule in their cytoplasm that is secreted, in most cases, as either a high- or a low-mol-wt BCGF molecule. This model implies that the neoplastic B cells possess functional receptors for the BCGF molecule that do not require in vitro activation in freshly prepared tumor cells. This type of autocrine stimulation also implies that the gene for BCGF, which does not appear to be expressed in normal, nonvirally transformed B cells, is expressed in neoplastic B cells such as HCL. Neoplastic B cell proliferation driven by an autostimulatory growth factor should circumvent the control mechanisms regulating normal B cell proliferation. This model appears to be consistent with the growth kinetics of most B cell neoplasms and suggests a number of interesting possible approaches to the control of neoplastic B cell growth in the future.

ACKNOWLEDGMENT

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Defective T Cell Responsiveness in Chronic Lymphocytic Leukemia: Analysis of Activation Events

By Neil E. Kay and Manuel E. Kaplan

Chronic lymphocytic leukemia (CLL) is a B cell disorder in which major T cell proliferative defects are present. We investigated the nature of this deficit by studying several parameters known to be crucial in normal T cell proliferative response to mitogen. Purified peripheral blood T cells from B-CLL patients were analyzed for the presence of T3 antigen. We observed that CLL T cells have a direct correlation between levels of T3 membrane antigen and proliferative response to mitogen. The appearance of activation antigens (transferrin, HLA-DR, and interleukin 2 [IL 2] receptor) was normal in CLL T cells post-mitogen exposure. Despite the normal presentation of IL 2 receptor on CLL T cell membrane, there was decreased production of IL 2 by CLL patients (*v* controls) (39.6 ± 10.2 cells per milliliter *v* 64.6 ± 11.0 cells per milliliter). Finally, we were able partially, but not fully, to reconstitute CLL T proliferative response to mitogen by adding purified exogenous IL 2. These findings suggest that CLL T cells have multiple defects that may impact on their proliferative potential. Further insight into these deficits may result in strategies that will facilitate immunologic restoration in T cells of these patients.

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CHRONIC LYMPHOCYTIC LEUKEMIA (CLL), typically a monoclonal B cell malignancy, is frequently accompanied by various peripheral blood T cell abnormalities.[1-8] These include T cell lymphocytosis,[1,2] low T4-T8 ratios with inadequate helper activity for B cell proliferation and immunoglobulin synthesis,[3-6] and depressed to absent natural killer activity[7,8] and autologous mixed lymphocyte reactivity.[9] In addition, variably decreased responses to mitogen-induced (phytohemagglutinin [PHA] or concanavalin A [Con A]) proliferation have been reported by several investigators.[10-12] We have recently described a subgroup of CLL patients in whom only a minority of erythrocyte rosette positive (ER$^+$) peripheral blood T cells reacted with monoclonal antibodies to T3,[12] a 20- to 25-kd membrane antigen normally present on mature T cells and medullary thymocytes.[13,14] In contrast to other CLL patients whose T3-ER$^+$ ratios were more normal (≥ 0.6), T cells from this CLL subgroup exhibited markedly defective responses to PHA and Con A. The T3 antigen is now known to be required for normal T cell activation.[15,16] Consequently, we have attempted to better define the mechanisms of abnormal T cell responsiveness to lectins in this group of CLL patients by enzymatically exposing additional membrane T3, studying the appearance of membrane activation antigens, measuring interleukin 2 (IL 2) production, and enhancing proliferative responses with exogenous normal IL 2.

MATERIALS AND METHODS

Eleven B-CLL patients and 11 normal donors, matched for age and sex, were studied. CLL patients were staged according to the method of Rai: stages 0 to 1, five patients; stage 2, four patients, and stage 4, two patients. No patient had received chemotherapy within four weeks of study.

Preparation of ER$^+$ cells. T cells were isolated by virtue of their ability to rosette with sheep erythrocyte (SRBC). Peripheral blood mononuclear cells were prepared from heparinized venous blood by Ficoll-Hypaque centrifugation, resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS), and depleted of adherent cells by incubation in sterile plastic Petri dishes for one hour. Nonadherent cells were incubated with SRBC pretreated with AET (2-aminoisoethylisothyronium hydrobromide), and the ER$^+$ cells were pelleted by centrifugation through Ficoll-Hypaque. ER$^+$ lymphocytes were freed of SRBC by brief hypotonic lysis in distilled water. This procedure gave a purity 98% or greater for ER$^+$ lymphocytes (measured by rerosetting with SRBC-AET).

Treatment of ER$^+$ lymphocytes with neuraminidase. ER$^+$ cells (2×10^7/mL) suspended in PBS were incubated with *Vibrio cholerae* neuraminidase (50 U/mL) (Behring Diagnostics, Somerville, NJ) at 37 °C for 30 minutes with shaking. The cells were then washed repeatedly with PBS to remove excess enzyme and resuspended in RPMI-10% FBS.

Detection of T cell membrane antigens. The following monoclonal antibodies were used to detect surface membrane antigens of ER$^+$ lymphocytes: OKT3, TAC (anti-IL 2 receptor), OKT9 (anti-transferrin receptor), and 5/12 (anti-IA). (Monoclonal antibodies OKT3 and OKT9 were purchased from Ortho Laboratories, Raritan, NJ; TAC was generously provided by Dr Thomas Waldmann, National Institutes of Health; 5/12 was a generous gift of Dr Soldano Ferrone, New York Medical College, NY.) ER$^+$ cells (1×10^6/mL) in RPMI-10% FBS were incubated (4 °C, 30 minutes) with 20 to 50 μL of an appropriate dilution of monoclonal antibody. The cells were then washed twice with RPMI-10% FBS and incubated for an additional hour with 100 μL of a 1:40 dilution of fluoresceinated goat F(ab)$'_2$ antimouse immunoglobulin (Dako, Burlingame, Calif). After thorough washing, the cells were examined with a Zeiss fluorescence microscope equipped with epifluorescence filters.

PHA-induced mitogenesis. ER$^+$ cells (2×10^5) isolated from controls or CLL patients were mixed in microtiter wells (Linbro, Hamden, Conn) with 1 μg/mL purified PHA (PHA-16, Burroughs Wellcome, Triangle Park, NC). After 72 hours of incubation (37 °C, 5% CO_2), 2 μCi of tritiated thymidine (^{3}[H]Tdr) (New England Nuclear, Boston) was added. After an additional 24-hour incubation, cells were harvested and radioactivity measured by liquid scintillation spectrometry. Results were calculated and expressed as mean ± standard deviation of the mean (SEM). In some studies, exogenous IL 2 was added simultaneously with PHA. The IL 2 was derived from normal, PHA-stimulated lymphocytes (see below), or recombinant IL 2 (generously provided by Cetus Corporation, Emeryville, Calif) was used. IL 2 concentrations ranging from 0.1% to 20% were used.

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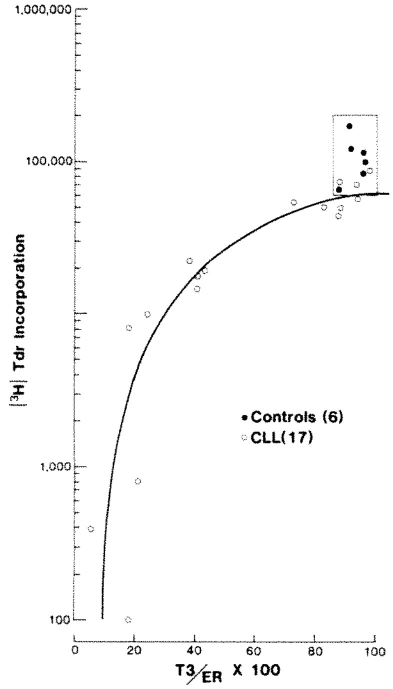

Fig 1. Relationship between T cell proliferation and T3 membrane antigen. The T3-ER$^+$ ratios of CLL (O) (n = 17) or control (●) (n = 6) subject lymphocytes are plotted *v* the ^{3}[H]Tdr incorporation of the same lymphocytes PHA-induced ^{3}[H]Tdr incorporation.

IL 2 production and quantitation. Lymphocytes (10^7/mL, control and CLL) in RPMI–10% FBS were incubated with 1 μg/mL PHA for 24 hours (37 °C, 5% CO_2). PHA–lymphocyte-conditioned media (PHA–LCM) supernatants were harvested and stored at −70 °C until being assayed for IL 2 activities. IL 2 measurements were performed by a modification of the method of Gillis et al,[17] using the CTLL-20, IL 2-dependent cell line.[18] CTLL-20 cells were maintained in RPMI–5% human serum and were fed every other day with 10% to 20% of a murine IL 2 source (spleen LCM derived from MLC). CTLL-20 cells were washed twice to remove residual IL 2, distributed into microtiter wells (0.5 to 1.0 × 10^4 CTLL per well), and supplemented with various dilutions of normal or CLL PHA–LCM. Dilutions of a standard human IL 2 source were invariably included. After 24-hour incubation, the cells were pulsed for six hours with 2 μCi ^{3}[H]Tdr. A log probit plot of the standard IL 2 source was used to quantify IL 2 activities of the unknown PHA–LCM.

RESULTS

Relationship between CLL T cell proliferation and membrane T3 antigen. We have previously reported that CLL T cell populations containing low T3-ER$^+$ ratios exhibit subnormal mitogenic responses to PHA.[12] The present studies confirm our previous findings and demonstrate a high degree of correlation between T3-ER$^+$ ratios and levels of ^{3}[H]Tdr incorporation (Fig 1). Ratios $\geq$0.6 were invariably associated with more normal proliferative responses. Patients with ratios $\leq$0.3 (n = 5) had significantly less proliferation than patients with ratios $\geq$0.6 ($P < 0.5$; n = 10).

In an effort to augment the defective responses, we treated CLL cell populations having low T3-ER$^+$ ratios with neuraminidase, which, as we have previously shown, enhances their T3 antigen expression.[12] Although neuraminidase treatment markedly increased the percentages of T3-positive cells from 38% to 78%, no significant improvement in PHA-induced ^{3}[H]Tdr incorporation resulted.

Appearance of membrane activation antigens in CLL and control T cells. Figure 2 summarizes the kinetics of appearance of various activation antigens after exposure of CLL and control T cells to PHA. During the first 48 hours, T9, TAC, and IA increased at similar rates in patient and control cells; thereafter, T9 and TAC gradually declined. In contrast, IA expression of control, but not of CLL, cells continued to increase between 48 and 72 hours. The rate and degree of expression of activation antigens bore no relationship to T3-ER$^+$ ratios or to the magnitude of ^{3}[H]Tdr incorporation. The standard error of the mean for all points plotted in Fig 2 ranged from 4% to 10%.

IL 2 production by CLL and control T cells. CLL T cells were found to elaborate approximately 55% of control IL 2 activities in response to PHA stimulation. Table 1 summarizes the relationship of CLL ER$^+$ lymphocytes to their membrane T3 presentation and PHA-induced IL 2 production and ^{3}[H]Tdr incorporation (Table 1). In general, the lower the T3-ER$^+$ ratio, the lower the thymidine incorpora-

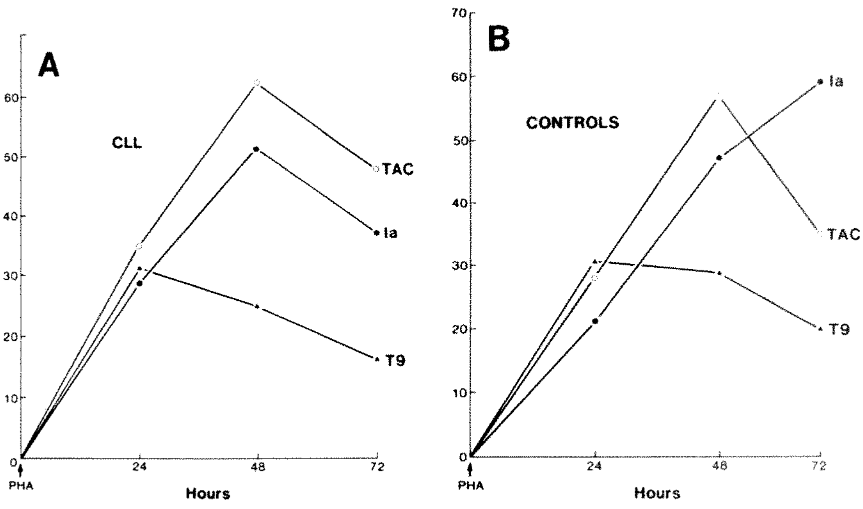

Fig 2. Sequential analysis of (A) CLL (n = 7) or (B) control (n = 7) ER$^+$ lymphocyte membrane activation and post-PHA exposure. The mean percentage of each activation antigen (TAC = O, Ia = ●, transferrin = ▲ T9) is plotted over a 72-hour incubation period.

Table 1. CLL ER$^+$ Lymphocytes in Relationship to Their T3 Membrane Antigen, IL2 Production, and PHA-induced ^{3}[H]Tdr Incorporation

	ER$^+$ Lymphocytes		
Group	T3 Positive (%)	IL 2 Production (μ/mL)	^{3}[H]Tdr (cpm/10^5 cells)
I (0%–30%, n = 4)	13.5 ± 2.9	31.5 ± 27.8	$2.9 \pm 1.8 \times 10^{-2}$
II (30%–60%, n = 4)	40.8 ± 1.0	32.8 ± 26.5	$18.5 \pm 1.4 \times 10^{-3}$
III (>60%, n = 6)	87.5 ± 3.6	45.8 ± 11.7	$59.3 \pm 6.8 \times 10^{-3}$
Control (n = 11)	92.5 ± 2.6	64.6 ± 11.0	$109.8 \pm 15.8 \times 10^{-3}$

CLL ER$^+$ lymphocyte groups were arbitrarily divided into three groups: I, ER$^+$ lymphocytes that were 0% to 30% reactive with OKT3; II, ER$^+$ lymphocytes 30% to 60% reactive with OKT3; and III, ER$^+$ lymphocytes >60% reactive with OKT3.

tion. IL 2 production was somewhat lower in ER$^+$ lymphocytes with <60% T3 reactive cells, but the difference was not statistically significant.

Enhancement of mitogenesis by exogenous IL 2. Because after PHA stimulation CLL T cells developed various activation antigens normally but produced diminished amounts of IL 2, we attempted to improve their mitogenic responsiveness by supplementing them with exogenous IL 2 (Fig 3). Addition of 10% IL 2 improved somewhat, but did not normalize, the proliferative responses of CLL T cells to PHA. The greatest degrees of improvement were seen in CLL patients exhibiting low T3-ER$^+$ ratios. Addition of higher concentrations of IL 2, or repetitive supplementation with IL 2 over the 72-hour incubation period, failed to further enhance T cell proliferative responses.

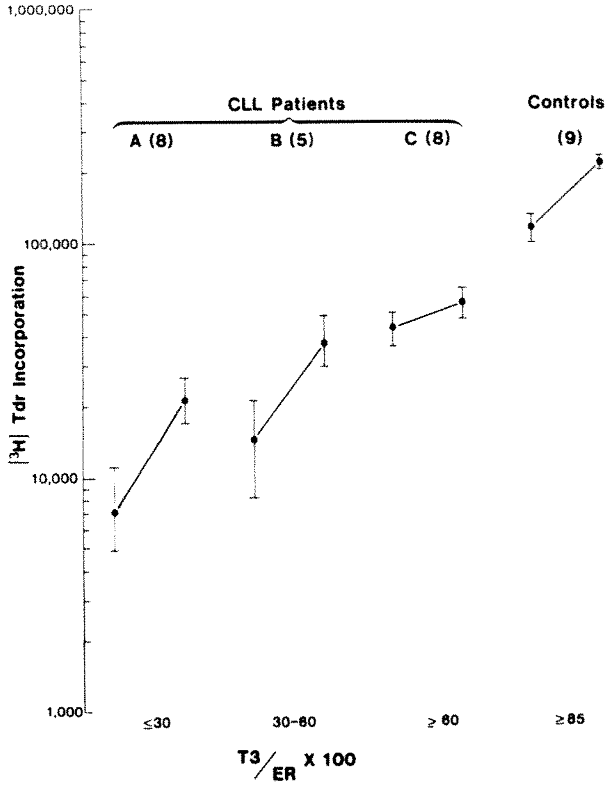

Fig 3. ^{3}[H]Tdr (mean ± SEM) incorporation of ER$^+$ lymphocytes pre- and post-exposure to 10% IL 2 are plotted for CLL and controls. CLL patients were subdivided into three groups based on the percentage of ER$^+$ cells that are T3 reactive: A = 0% to 30% T3$^+$, B = 30% to 60% T3$^+$, and C = >60% T3$^+$.

DISCUSSION

Activation of normal T cells may be arbitrarily conceptualized as occurring in three sequential stages: (1) engagement of Ti, the T cell antigen receptor intimately associated with T3[19]; (2) development of multiple membrane activation antigens, including T9 (transferrin receptor), TAC (the receptor for IL 2); the T cell growth factor, and IA[20]; (3) synthesis and elaboration of IL 2, which combines with TAC to trigger mitogenesis.[21] In an effort to better understand the mechanisms of the defective proliferative responses of CLL T cells to PHA and other immunologic stimuli, we have examined each of the stages for possible abnormalities. We have previously reported that the peripheral blood of CLL patients showing exceedingly poor responses to PHA contain high percentages of ER$^+$ cells that poorly express T3 membrane antigen.[12] T3 is known to be required for normal T cell proliferative responses and development of cytotoxic effector cell functions.[15,16] The studies described confirm our previous findings and emphasize the close relationship between T3 expression and mitogenic responsiveness (Fig 1). Although treatment of these low T3-ER$^+$ cell populations with neuraminidase significantly improved their T3 expression, no parallel enhancement of mitogenic responsiveness occurred. The T3 antigen comprises several membrane glycoproteins containing different numbers of sialic acid residues.[14] Because neuraminidase treatment exposes additional T3 antigenic sites without enhancing mitogenic responsiveness, additional yet undetected defects may be present within the CLL T3 complex. Alternatively, other abnormalities may be responsible for defective cell proliferation.

To investigate other possible abnormalities, we studied the appearance of various membrane activation antigens induced by PHA stimulation of CLL T cells. Surprisingly, the rates of appearance of T9, TAC, and IA were entirely normal, unrelated to the degree of T3 expression or to the mitogenic responsiveness of the individual cell populations. Although we detected significantly diminished IL 2 activity in PHA–CLL T cell conditioned media, no relationship was observed between the degree of abnormality in IL 2 elaboration, T3-ER$^+$ ratios, and mitogenic responsiveness. Moreover, only minimal improvement in the proliferative responses of these cells to PHA could be induced by addition of exogenous IL 2, despite the presence of apparently normal numbers of IL 2 receptors. These findings suggest that receptor binding of IL 2 may be defective or that even more distal defects in the mitogenic sequence may be present, ie, in the generation of the recently described inducible cytoplasmic factor that initiates DNA replication.[22]

Thus it appears that CLL T cells exhibit multiple abnormalities that may relate to their diminished proliferative potential. Continued exploration of the molecular nature of these abnormalities should yield insights that may permit immunologic reconstitution of patients with this disorder.

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The Detection of Specific Antibody Formation to Recall Antigens After Human Bone Marrow Transplantation

By Lawrence G. Lum, Nathan A. Munn, Moses S. Schanfield, and Rainer Storb

The results of this study show that donor-derived immunity can be detected and persists in long-term survivors with and without chronic graft-*v*-host disease (GVHD) after human marrow grafting. Seventy-one marrow recipients (60 long-term and 11 short-term survivors) were studied for the presence of specific serum IgG antibodies to tetanus toxoid (TT), and 46 marrow recipients (35 long-term and 11 short-term) were tested for antibodies to diphtheria toxoid (DT) and measles virus after marrow grafting using an enzyme-linked immunosorbent assay. Of the 60 long-term survivors, 31 were healthy and 29 had chronic GVHD. None of the recipients were immunized to the test antigens postgrafting. Most long-term healthy recipients exhibited antibody titers to the recall test antigens, whereas only a minority of those with chronic GVHD had antibody titers to recall antigens. In healthy long-term recipients (≥ one year postgrafting) whose donors were immune to the test antigens, 25 of 31 had titers to TT, 11 of 17 had titers to DT, and 12 of 20 had titers to measles. In recipients with C-GVHD, 13 of 29 had titers to TT, six of 15 had titers to DT, and six of 15 had titers to measles virus. Within 100 days postgrafting, 11 of 11 had anti-TT titers, ten of ten had anti-DT titers, and seven of eight had antimeasles virus titers.

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EARLY EXPERIMENTS in mice and rats showed that the infusion of bone marrow, spleen cells, lymph node cells, or thymus cells from an immune donor could transfer specific immune reactivity.[1-4] However, such adoptive transfer experiments showed only transient antibody-forming ability in the recipients. Studies in the canine marrow-grafting model showed that certain canine marrow recipients exhibited distemper titers after DLA-matched marrow transplantation without posttransplant immunizations to distemper.[5] Because both the marrow donors and the recipients were immunized to distemper before marrow transplantation, the interpretation of such data was difficult.

Despite a large body of literature on immune reconstitution after human marrow transplantation (reviewed in ref. 6), information on the transfer of specific immunity from donors to recipients after human marrow transplantation is scarce. Earlier studies examined recipient's delayed hypersensitivity responses to recall antigens and antibody responses to keyhole limpet hemocyanin, bacteriophage ΦX174, and pneumococcal polysaccharide antigens.[7-10] These studies showed primary and secondary responses to neoantigens by one to two years postgrafting. These studies did not address the question of transfer of specific antibody-forming ability from marrow donors to marrow recipients. One early report used keyhole limpet hemocyanin immunization of a twin donor as an immunologic marker for bone marrow graft acceptance in an identical twin transplanted for acute lymphocytic leukemia.[11]

This study investigates the transfer of specific antibody-forming ability from the marrow donor to the marrow recipient. The investigation shows that there is rapid development of this ability by recipient lymphocytes without postgrafting immunization to tetanus toxoid (TT), diphtheria toxoid (DT), and measles virus when the donor lymphocytes were immune to these antigens. The donor-derived immunity can be detected and persists in long-term survivors with and without chronic graft-*v*-host disease (GVHD). Such data on these common recall antigens will facilitate the development of immunization recommendations for the management of transplant recipients.

MATERIALS AND METHODS

Subjects. Seventy-one patients transplanted for hematologic malignancy or aplastic anemia were studied for the presence of serum antibody titers. Sixty-five received allogeneic transplants from HLA-identical siblings, five received syngeneic transplants, and one received an HLA-haploidentical transplant. In this study there were 14 chronic myelogenous leukemia, 15 acute lymphocytic leukemia, 27 acute nonlymphocytic leukemia, two Hodgkin's lymphoma, one erythroleukemia, one lymphatic leukemia, and 11 aplastic anemia patients. Patients with hematologic malignancies were conditioned with cyclophosphamide and total body irradiation[12-14] and patients with aplastic anemia were conditioned only with cyclophosphamide.[15] Methotrexate, cyclosporine, or both were used as posttransplant prophylaxis for GVHD.[16] Cyclosporine or antithymocyte globulin was used for the treatment of established acute GVHD, while others received steroids or a combination of the above.

Twenty-nine long-term survivors (>100 days postgrafting) developed chronic GVHD, and 20 of these were treated with either prednisone alone or in combination with cyclophosphamide, procarbazine, or azathioprine. Thirty-one healthy long-term survivors were studied. Seventy-one patients were studied pretransplant and posttransplant for IgG anti-TT titers, and 46 of these were also studied for IgG anti-DT and IgG antimeasles titers. None of the recipients were immunized to the test antigens posttransplant. None of the long-term survivors who were studied at ≥ one year postgrafting had received blood products in the six-month interval before testing. The 60 long-term survivors were studied betwen 320 and 3,295 days postgrafting. Twenty-five of these were studied longer than two years postgrafting. The bone marrow donors did not receive booster immunizations to the test antigens in the two-year period before marrow donation.

Serum collection. Serum samples were collected from marrow transplant recipients and their donors pretransplant and on the days posttransplant as indicated in Figs 1 through 3.

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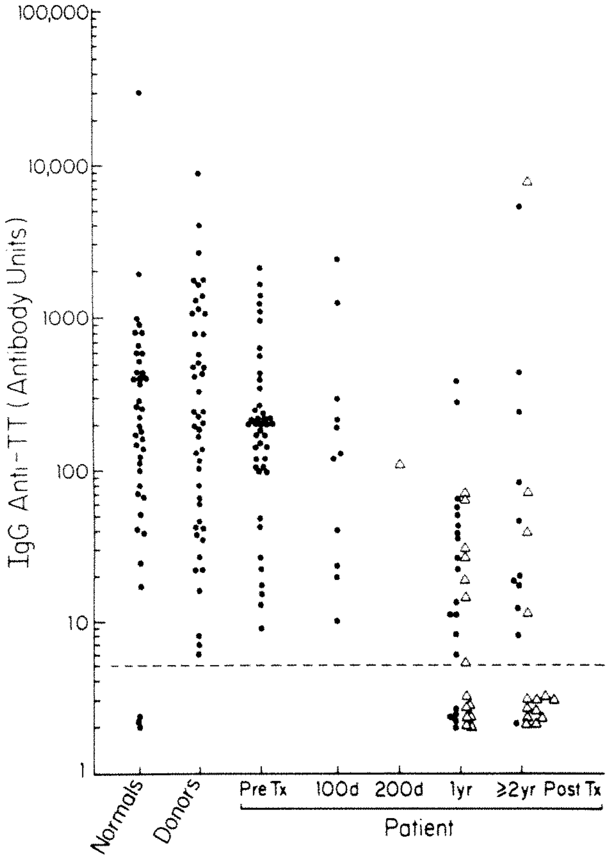

Fig 1. The IgG anti-TT titers (AU) are summarized for the normal controls, the donors, the pretransplant host, and the titers of the marrow-graft recipients with (Δ) and without (●) chronic GVHD. Each recipient was studied once postgrafting. Points below the dashed line were considered negative titers.

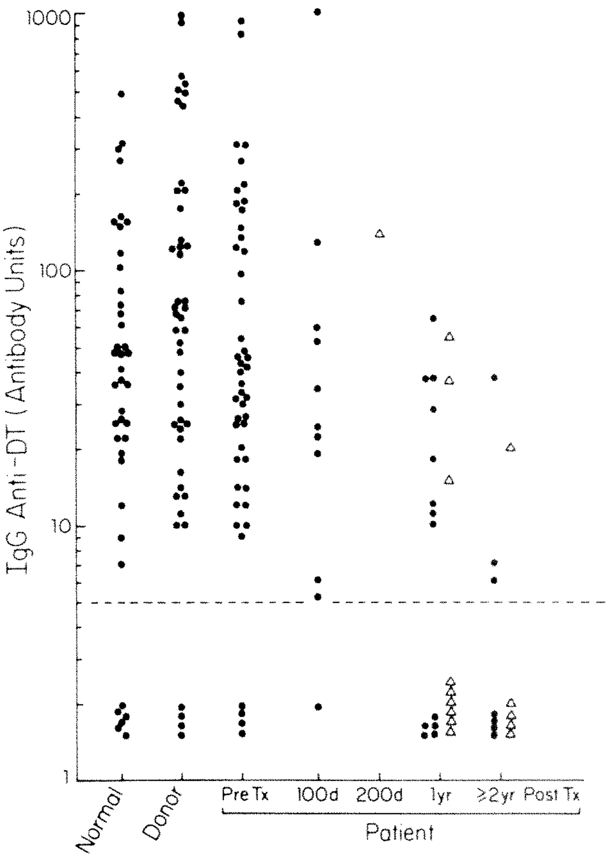

Fig 2. The IgG anti-DT titers (AU) are summarized for the normal controls, the donors, the pretransplant host, and the titers of the marrow-graft recipients with (Δ) and without (●) chronic GVHD. Each recipient was studied once postgrafting. Points below the dashed line were considered negative titers.

Cytogenetics. When there were differences in cytogenetic sex markers, all but one of the recipients (30 out of 31) had lymphocytes of donor origin. One individual was a mixed chimera. Cytogenetics on the remaining donor–recipient pairs were not informative.

Enzyme-linked immunosorbent assay (ELISA). Serum IgG titers to TT, DT, and measles were measured using an ELISA modified from that described.[17,18] Briefly, NUNC EIA plates (NUNC, Copenhagen, Denmark) were coated with 100 μL of either 1.0 Lf/mL TT or 2.0 Lf/mL DT (lots No. LP463P and DT254PD, respectively, Commonwealth of Massachusetts Department of Public Health, Boston), or a 1:100 dilution of measles antigen (lot No. X30-364, M.A. Bioproducts, Los Angeles) diluted in Tris buffer, pH 9.6, 15 to 18 hours at 37 °C.

Plates were washed in phosphate-buffered saline (PBS) containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20, Sigma Chemical Co, St Louis) and blocked for one hour at 37 °C with 10% bovine serum albumin in PBS. Plates were then washed in PBS–Tween solution and stored at −20 °C. Plates were warmed and washed in PBS–Tween solution before use.

Anti-TT IgG standard was prepared from high-titer anti-TT human serum, depleted of non-IgG protein by QAE-A50 Sephadex (Pharmacia, Piscataway, NJ) and affinity purified over TT-linked Sepharose 4B (Pharmacia). The amount of specific IgG anti-TT antibody in the standard was directly calibrated in a polyclonal IgG ELISA using goat antihuman IgG specific underlayer (capture) antibody (Tago, Burlingame, Calif) and a peroxidase-conjugated goat antihuman IgG developer.[18]

The antibody concentrations of unknown serum samples diluted on a $\log_{10}$ basis for IgG anti-TT, IgG anti-DT, and IgG antimeasles were interpolated directly from the affinity-purified IgG anti-TT standard.

The optical density reading of the last positive serum dilution was compared with the linear portion of the linear regression line drawn from the human IgG anti-TT standard.[18] Only optical density values between 0.1 and 1.0 were used for interpolation. Known dilutions of the anti-TT standard were placed into triplicate wells coated with TT on each plate. Therefore, IgG antibody units (AU) relative to the IgG anti-TT standard could be interpolated directly from the optical densities of the IgG anti-TT standard on each plate for DT and measles virus titers with 1 AU ≈ dilution of serum × 1 ng/mL estimated by direct comparison with the IgG anti-TT standard. The goat antihuman IgG-specific peroxidase-conjugated developer would bind only with IgG bound to the specific antigen on the plate. The IgG-specific developer did not detect IgA, IgM, or IgE standards at the upper concentration of 20 ng/mL.

One hundred microliters of diluted serum was added to each microtiter well. All dilutions were done in 10% fetal calf serum in balanced salt solution. The plates were incubated for two hours at 37 °C and washed with PBS–Tween solution. One hundred microliters of peroxidase-conjugated goat anti-human IgG antibody (lot No 901401, Tago) diluted 1:1,500 in 10 mmol/L phosphate buffer was

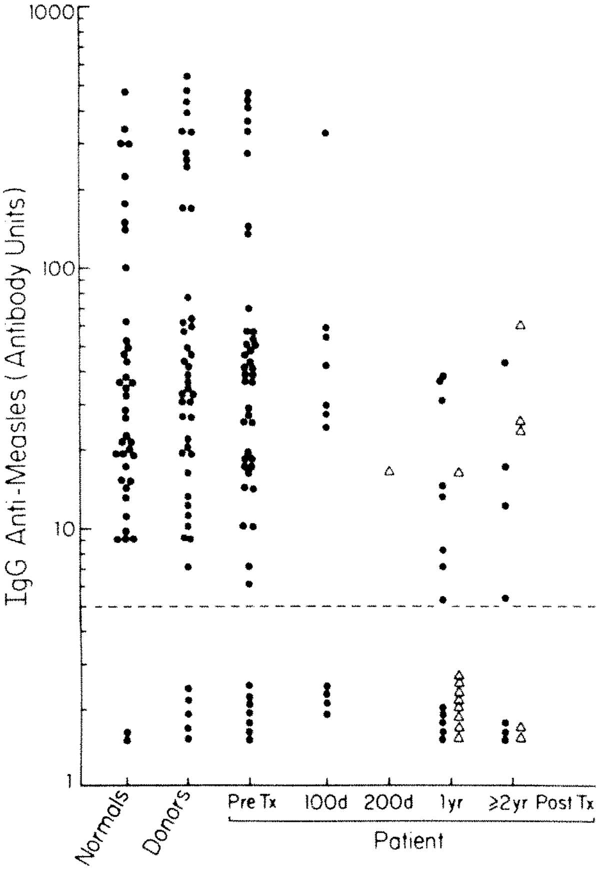

Fig 3. **The IgG antimeasles titers (AU) for the normal controls, the donors, the pretransplant host, and the titers of the marrow-graft recipients with (Δ) and without (●) chronic GVHD. Each recipient was studied once postgrafting. Points below the dashed line were considered negative titers.**

added to each well, incubated at 37 °C for one hour, and washed with PBS–Tween. One hundred microliters of a 1.0 mg/mL solution of 2,2′-azinodi-(3-ethylbenzthiazoline sulfonic acid) in citrate buffer, pH 4, plus 0.03% H_2O_2 was added to all wells. The optical density was read at 414 nm after 30 minutes.

An optical density equal to 0.1 at a 1:1,000 serum dilution or above was considered a positive titer. Backgrounds were infrequently present at serum dilutions ≤1:100. Serum from agammaglobulinemic patients and from normal individuals known by history to be negative for a specific antigen had an optical density <0.1 at a serum dilution of 1:100.

Normal range for antibody titers. Forty normal individuals and 71 marrow donors ranging in age from 3 to 50 years served as controls to determine the normal interval. Normal range was defined as the interval above which 95% of the immune normal individuals studied had specific IgG titers to TT, DT, and measles virus. Titers below this interval were considered negative.

Statistics. For all analyses, the antibody titers were compared in a Wilcoxon rank sum test (two-tailed). For DT and measles titers, four and five patients, respectively, had donors who did not have titers and these were excluded from the statistical analysis, although they are shown in the figures. All donors had TT titers.

RESULTS

Antibody titers to TT. Figure 1 summarizes the serum IgG anti-TT titers in AU for the normal controls, the marrow donors, and the marrow recipients pretransplant and posttransplant. Each marrow graft recipient was studied only once after marrow grafting. The study was not sequential. Figure 1 shows IgG anti-TT titers on healthy recipients and those with chronic GVHD. All of their donors had normal IgG anti-TT titers. Between one and two years, 15 of 20 (75%) healthy survivors and seven of 14 (50%) recipients with chronic GVHD had anti-TT titers. After two years, ten of 11 (91%) healthy survivors and five of 15 (33%) recipients with chronic GVHD had anti-TT titers. Statistical compari-

Table 1. Analysis of Patient–Donor Pairs Pretransplant and Posttransplant

Immune Status of Patient–Donor Pairs	IgG Anti-TT Ab (n = 50)		IgG Anti-DT Ab (n = 45)		IgG Anti-measles Ab (n = 45)	
	Healthy	C-GVHD	Healthy	C-GVHD	Healthy	C-GVHD
Informative for specific immunity						
Donor (+)						
Patient pre-tx (−)	0	0	0	1	2	0
Patient post-tx (+)						
Donor (−)						
Patient pre-tx (+)	0	0	2	0	1	0
Patient post-tx (−)						
Donor (+)						
Patient pre-tx (+)	31	10	21	5	17	5
Patient post-tx (+)						
Noninformative for specific immunity						
Donor (−)						
Patient pre-tx (−)	0	0	2	0	4	0
Patient post-tx (−)						
Donor (+)						
Patient pre-tx (+)	2	7	6	7	5	10
Patient post-tx (−)						
Donor (+)						
Patient pre-tx (−)	0	0	0	1	1	0
Patient post-tx (−)						

A (+) signifies a titer ≥1:1,000, a (−) signifies a titer <1:1,000 by ELISA. The combination of donor (−), patient pre-tx (+), and patient post-tx (+) did not occur. Pre-tx, pretransplant; post-tx, posttransplant.

sons between the healthy long-term chimeras and the marrow-graft recipients with chronic GVHD show that the healthy chimeras had significantly higher IgG anti-TT titers than their counterparts with chronic GVHD ($P < .006$).

Antibody titers to DT. Figure 2 summarizes the serum IgG anti-DT in AU for the normal controls, the marrow donors, and the marrow recipients pretransplant and posttransplant. Four donors did not have detectable IgG anti-DT antibody titers. Each transplant recipient was studied once postgrafting. Ten of ten (100%) recipients 100 days postgrafting had anti-DT titers in the normal range. Between one and two years, eight of 11 (73%) healthy survivors and three of nine (33%) recipients with chronic GVHD had anti-DT titers. After two years, three of six (50%) healthy survivors and two of six (33%) recipients with chronic GVHD had anti-DT titers. There was no statistical difference between recipients with and without chronic GVHD.

Antibody titers to measles virus. Figure 3 summarizes the serum IgG antimeasles virus in AU for the normal controls, the marrow donors, and the marrow recipients pretransplant and posttransplant. Five donors did not have detectable IgG antimeasles virus antibody titers. Each transplant recipient was studied once postgrafting. Seven of eight (88%) recipients had antibody titers to measles virus 100 days postgrafting. Eight of 12 (67%) healthy survivors and one of nine (11%) recipients with chronic GVHD had antimeasles virus titers between one and two years after marrow grafting. After two years, four of six (67%) healthy survivors and four of six (67%) recipients with chronic GVHD had antimeasles virus titers. There was no statistical difference between the healthy long-term survivors and those with chronic GVHD.

Chronological recovery of specific antibody titers in a single patient. One recipient (UPN 1353) with chronic GVHD was studied 791 and 1,061 days postgrafting. She received an HLA-identical, mixed lymphocyte culture nonreactive marrow graft from her brother. The recipient had a measles virus titer of 23 AU/mL and had no titers to TT or DT 791 days postgrafting. By 1,061 days postgrafting, the recipient had 211 AU of IgG antimeasles virus, 13 AU of IgG anti-TT, and 27 AU of IgG anti-DT. Cytogenetic sex markers on both occasions showed the lymphocytes to be of donor (XY) origin. Both donor and recipient were immune to all three antigens pretransplant.

Evidence for transfer of specific antibody immunoreactivity in donor–recipient pairs. Data in Table 1 summarize the titer data for all recipients. Table 1 contains both informative and noninformative data; the data shown in boldfaced type are informative. Table 2 gives the specific details of the six informative cases in boldfaced type selected from Table 1. In three of the six cases shown, donors had measurable antibodies to measles or diphtheria antigens, while recipients were negative before transplantation. When tested 80 to 465 days after transplantation, all three recipients made antibodies presumably through donor-derived cells. Conversely, three donors did not make antibodies to measles or diphtheria antigens, while their respective recipients did when tested before grafting. When tested 385 to 616 days after grafting, all three recipients failed to make antibody, signifying the disappearance of host-derived immunity and the establishment of donor cells not previously exposed to these antigens.

Table 2. Evidence for Transfer of Specific Immunity

UPN	Antigen	Donor Titer	Recipient Titers Pregrafting	Recipient Titers Postgrafting	Day of Study
2268	Measles	30	—	24	80
1668	Measles	38	—	30	465
1854	Measles	—	38	—	385
1782	Diphtheria	539	—	15	318
1823	Diphtheria	—	18	—	426
1502	Diphtheria	—	33	—	616

The specific antibody titers are estimated antibody units (AU) as described in Materials and Methods. Specific antibody titers to measles or DT ≤5.0 AU were considered negative. The last column shows the number of days postgrafting that serum was obtained. A minus (−) indicates a titer below the normal range.

DISCUSSION

The assumption that antibody production to diphtheria and measles beyond day 100 after allogeneic bone marrow transplantation represents immunity transferred with donor cells is based on at least four pieces of evidence: (1) recipients with antibody production after bone marrow transplantation had donors immune to the same antigens pretransplant; (2) when donors failed to make antibodies, recipients were found to be negative after bone marrow transplantation, including recipients who made antibodies before bone marrow transplantation; (3) immunoglobulin allotype production of host type ceases by day 120 after bone marrow transplantation in 97% of the recipients[19]; and (4) in recipients with ABO-incompatible transplants, host-type isohemagglutinin production ceases at a median of 40 days after bone marrow transplantation, although immunity may persist in isolated cases for up to five months postgrafting.[9]

The majority of long-term healthy recipients developed serum IgG antibody titers to TT, DT, and measles. It is not likely that these findings represent environmental re-immunization, particularly in the short period of time postgrafting. Although one study has shown mixed chimerism in the lymphocytes of two marrow graft recipients and host-type RBC markers more than one year postgrafting,[20] it is unlikely that host-type B cells could account for the normal levels of IgG anti-TT, anti-DT, and anti-measles antibodies in so many marrow-graft recipients. Marrow-graft recipients who did not develop titers to TT, DT, or measles virus posttransplant show that the transfused antibody did not contribute to antibody titers postgrafting.

Most marrow-graft recipients with chronic GVHD failed to develop antibody titers to recall antigens. The reasons for failure to develop specific antibody titers in this group of recipients are not clear. Both chronic GVHD and the treatment thereof with immunosuppressive regimens could suppress the development of antibody-forming cells.

Immunoglobulin allotyping confirmed donor-origin Ig allotypes in two donor–recipient pairs who were confirmed to be IgG allotype mismatched; however, we were not technically able to type affinity-purified IgG anti-TT–specific antibodies from these individuals to show allotype transfer of IgG anti-TT directly.

Mature T and B cells transferred in the marrow inoculum were probably responsible for producing specific antibodies. Recent studies using a TT-stimulated specific antibody synthesis showed that purified T cells from long-term marrow-graft recipients can help IgG anti-TT antibody production in vitro after stimulation with TT in cocultures with immune donor B cells[21] and that recipient B cells can produce IgG anti-TT in the presence of donor helper T cells. Our findings are consistent with a recent study using human bone marrow obtained after primary and booster immunizations with TT that showed B cells could spontaneously synthesize IgG anti-TT and could be stimulated with pokeweed mitogen to produce IgG anti-TT.[22] The presence of marrow mononuclear cells capable of producing IgG anti-TT in immune subjects and the presence of antigen-specific lymphocytes in the peripheral blood of long-term marrow recipients support the contention that mononuclear cells in the marrow inoculum are responsible for the transfer of immunity.

Persistent antigen in the reticuloendothelial system did not play a major role in the re-presentation of antigen to newly maturing cells from the transplanted marrow, since immune recipients who received marrow grafts from nonimmune donors did not develop specific antibody titers.

In the first 100 days, persistent differentiated host B cells and passive transfusion of antibody may contribute to detectable antibody. However, the levels of specific antibody detected at 100 days postgrafting suggest a major contribution by B cell engraftment, since it would be nearly impossible to attain normal antibody levels from packed red cells and/or platelet transfusions.

Insufficient numbers of donor–recipient pairs prevented an analysis of factors that might influence the formation of specific antibody, such as the relationship between identical twin transplants and healthy allogeneic recipients, the preparative regimen, the pretransplant diagnosis, and the type of posttransplant immunosuppression.

These results help to explain why more transplant recipients do not develop life-threatening infections with diseases against which their donors had been immunized. In more than 1,500 patients transplanted in our center, only one case of measles has occurred. A recipient developed measles after being exposed to his donor who developed measles postgrafting. In this case, the donor was probably not immune to measles pretransplant. These clinical observations suggest that many recipients are indeed protected by specific antibodies to recall antigens produced by donor-derived B cells. If transfer of antibody-forming capability to viruses occurred as satisfactorily as transfer of specific antibody-forming capabilities to measles, immunity to mumps, rubella, and polio may be transferred equally well. However, only followup on marrow recipients will determine whether their circulating levels of specific antibody are protective.

The proportion of marrow-graft recipients developing specific antibody titers or the levels of specific antibody might be increased by booster immunizations to the donor pretransplant. Healthy recipients or recipients with chronic GVHD who have not developed specific antibody titers by one year after transplant should be immunized with appropriate vaccines and evaluated for specific antibody titers. Those with chronic GVHD may not respond to immunizations and healthy individuals may respond to recall antigens as if there were "neoantigen" antigens.

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Anti-CALLA Antibodies Identify Unique Antigens on Lymphoid Cells and Granulocytes

By John M. Pesando, Patricia Hoffman, Nancy Martin, and Ted Conrad

The common acute lymphoblastic leukemia antigen (CALLA) is a 100-kd surface glycoprotein that is present on normal and malignant lymphoid cells. It is a useful marker for distinguishing between clinically important types of acute leukemia. Anti-CALLA monoclonal antibodies (MoAb) also react with mature myeloid cells (granulocytes), where they identify an antigen having a similar molecular weight (mol wt). We now report that the antigens detected by anti-CALLA MoAb on human lymphoid and myeloid cells differ in their behavior and chemistry. Surface-labeling studies indicate that the antigen on lymphoid cells has a mol wt of approximately 100 kd v 110 kd for that on granulocytes. When cells are metabolically labeled with ^{35}S-methionine, differences in the mol wt of these antigens are again observed. Unlike the lymphoid antigen, expression of that on purified granulocytes is not modulated by incubation with specific antibody. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of proteolytic digests of the two antigens fails to clarify their chemical relationship. Thus the antigens detected on these two cell types may share an epitope(s) but be chemically distinct, or CALLA may exist in distinct forms and behave differently on lymphoid cells and granulocytes.
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CALLA IS A 100-kd surface glycoprotein expressed on malignant cells of approximately 70% of patients with acute lymphoblastic leukemia (ALL) and on rare lymphoid cells in normal bone marrow.[1-3] Its expression is reduced or modulated by incubation of cells with specific antibody.[4-7] CALLA is not expressed by leukemic cells of patients with acute nonlymphocytic leukemia (ANL) or of most patients with T cell ALL, making it a useful marker for diagnosing clinically important subtypes of acute leukemia. Its function remains unknown.

On normal cells, CALLA appears to be preferentially expressed on immature, rather than mature, lymphocytes. In contrast, anti-CALLA monoclonal antibodies (MoAb) react with mature (granulocytes) rather than immature myeloid cells, where they identify a 110-kd surface protein.[8-10] Study of the effects of anti-CALLA MoAb on the specialized functions of granulocytes might therefore clarify the role of this antigen. However, the results of our present serologic and immune precipitation studies indicate significant differences in the antigens identified on myeloid and lymphoid cells.

MATERIALS AND METHODS

Antibodies. Five CALLA-specific MoAb were used (anti-CALLA-1 (J5), anti-CALLA-4, anti-CALLA-5, anti-CALLA-6, and anti-CALLA-7).[10,11] Hybridomas producing these MoAb were raised by immunization of BALB/c mice with CALLA-positive malignant cells. Anti-CALLA-1 was raised to leukemic cells from a patient with ALL,[11] anti-CALLA-4, -5, and -6 to the ALL cell line Laz 221,[10] and anti-CALLA-7 to the Burkitts cell line Ramos. All identify a similar 100-kd surface glycoprotein and show identical reactivity patterns with cells from a panel of 38 acute leukemias and 32 hematopoietic cell lines. Modulation of CALLA by our initial anti-CALLA MoAb (J5) inhibits binding of the four other CALLA-specific MoAb.[4,5] MoAb were obtained from either cell culture supernates or ascites from tumor-bearing mice. They were ultracentrifuged and filtered before use.

Cells. Normal granulocytes were isolated by density gradient centrifugation on Ficoll-Diatrizoate (LSM, Litton Bionetics, Kensington, Md).[10] Wright-Giemsa staining confirmed that cell purity was greater than 90%. Viability was greater than 95%, as measured by trypan blue exclusion. Laz 221[12] and NALM-6[13] are CALLA-positive ALL cell lines.

Immunofluorescence analysis. Binding of MoAb to target cell populations at 4 °C was determined using a standard indirect immunofluorescence assay[2] and monitored on the fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Mountain View, Calif), with fluorescence displayed in the logarithmic mode. Before analysis, cells were suspended in RPMI 1640 containing 5% human AB serum to block Fc receptor sites. MoAb were used at a 1:100 dilution of ascites fluid to ensure antibody excess.

Antigenic modulation. Modulation of CALLA by specific MoAb was performed as described previously.[4,5] Filter-sterilized anti-CALLA or isotype-matched control MoAb (100 μg/mL per 1×10^6 cells) was added to appropriate cultures, followed by incubation at 37 °C for 16 hours. The extent of modulation was monitored by indirect immunofluorescence using the FACS.

Identification of antigens. Cell surface radiolabeling with ^{125}I and immune precipitation of detergent-solubilized cell extracts were performed using standard methods.[5] Antigens were resolved by electrophoresis on a 7% to 20% gradient sodium dodecyl sulfate-polyacrylamide slab gel (SDS-PAGE) and visualized by autoradiography. A ratio of 1 mCi of ^{125}I to 50×10^6 cells was used. For metabolic labeling, freshly purified granulocytes or NALM-6 cells were incubated with 1 mCi of ^{35}S-methionine per 50×10^6 cells for 16 hours at 37 °C in methionine-free media.[6] Detergent-solubilized cell extracts were purified by affinity chromatography on a lentil lectin column. Retained material was used in immune precipitation experiments following elution with alpha-methyl-D-mannoside.

Peptide analysis. Proteolytic digests of radiolabeled antigens identified by anti-CALLA MoAb on NALM-6 cells and granulocytes were compared using a modification of the method of Cleveland et al.[14] Nonreducing sample buffer was added to pellets of iodinated antigens purified by immune precipitation, followed by a three-minute incubation at 110 °C. Immediately before electrophoresis, samples were treated with fresh solutions of individual proteases in the same sample buffer or sham treated for 30 minutes, all at 22 °C. Proteolysis was terminated by addition of 2-mercaptoethanol to a final concentration of 5%, followed by a repeat three-minute

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incubation at 110 °C. Three proteases were used: trypsin, chymotrypsin, and papain (Sigma Chemical Co, St Louis).

RESULTS

Incubation of purified leukemic cells with specific antibody at 37 °C produces a cell population with a reduced capacity to bind anti-CALLA MoAb (antigenic modulation or, more correctly, epitope modulation) (Fig 1). In contrast, incubation of purified granulocytes from humans (nine donors) or from *Macaca fascicularis* (three animals) with anti-CALLA MoAb under identical conditions does not modulate expression of the detected epitope. Titration studies indicate that supernates from treated samples of both ALL cells and granulocytes contain comparable amounts of anti-CALLA MoAb, thus ruling out selective proteolysis of MoAb by the granulocyte sample. It is of note that the granulocyte antigen can be induced to modulate by anti-CALLA MoAb in the presence of monocytes (data not shown). This phenomenon has been observed with other antigens.[15,16]

Anti-CALLA MoAb identify a 100-kd glycoprotein on lymphoid cells metabolically labeled with ^{35}S-methionine or surface labeled with ^{125}I. In contrast, the molecular weight (mol wt) of the antigen identified by anti-CALLA MoAb on granulocytes from the same individual varies with the method of labeling used. Metabolic labeling with ^{35}S-methionine yields a 90-kd antigen, whereas surface labeling with ^{125}I identifies one of 110 kd (Figs 2 and 3). Although the mol wt of the antigen detected in biosynthetically labeled granulocyte preparations suggests that it is not made by contaminating lymphoid cells, it does not permit unequivocal identification of p90 as CALLA. Because p90 is not found on granulocytes that are surface labeled with ^{125}I, it may not be present or exposed on the cell surface. Further work is necessary to study these two forms of the granulocyte antigen.

To assess the homology of their polypeptide structures, proteolytic digests of the antigens identified by anti-CALLA-1 MoAb on iodinated human granulocytes and ALL cells were analyzed by SDS-PAGE and visualized by autoradiography (Fig 3). Proteolysis of the antigen isolated from ALL cells produces multiple novel lower mol wt species. Why several of the cleavage products generated by these three proteases have similar mol wt (most notably those at approximately 75, 55, and 45 kd) is unclear.

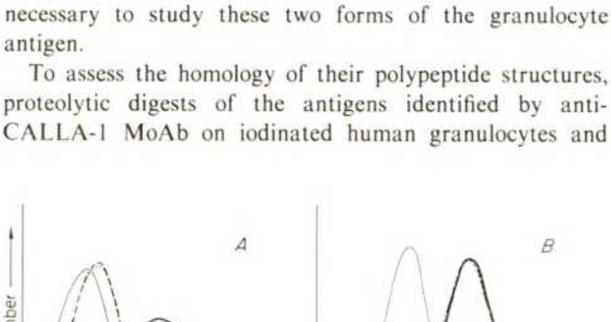

Fig 1. **Modulation of CALLA. Cells from the ALL cell line Laz 221 or purified granulocytes were incubated with comparable amounts of anti-CALLA-1 or an isotype-matched control MoAb for 16 hours at 37 °C (Panels A and B, respectively). They were then assayed for their ability to bind control or anti-CALLA-1 MoAb. Samples are as follows: control and anti-CALLA-1 MoAb on sham-modulated cells (thin and thick solid curves, respectively); control and anti-CALLA-1 MoAb on anti-CALLA-1 treated cells (thin and thick dashed curves, respectively). The ability to bind anti-CALLA-1 MoAb after incubation at 37 °C with the same reagent is decreased on ALL cells but not on granulocytes.**

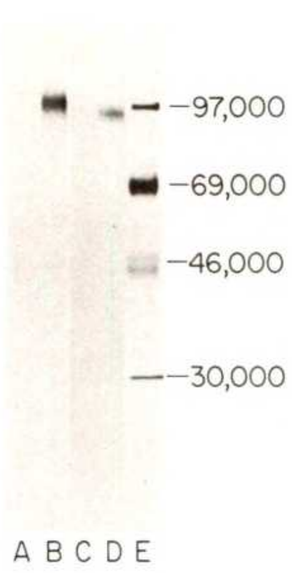

Fig 2. **Antigens detected by anti-CALLA-1 MoAb on ALL cells and granulocytes metabolically labeled with ^{35}S-methionine. Samples are as follows: A, control MoAb on NALM-6 cells; B, anti-CALLA-1 MoAb on NALM-6 cells; C, control MoAb on granulocytes; D, anti-CALLA-1 MoAb on granulocytes; and E, ^{14}C-labeled mol wt standards. Markers were purchased from New England Nuclear (Boston) and include phosphorylase B (97 kd), albumin (69 kd), ovalbumin (46 kd), and carbonic anhydrase (30 kd).**

Proteolysis of the granulocyte antigen reveals a different pattern. New radiolabeled bands are much less intense than those observed with the lymphocyte antigen. Although several new bands are seen with proteolysis of both antigens, others appear to be unique to the lymphocyte antigen (particularly those having higher mol wt). No new bands unique to the granulocyte antigen are observed. Results are unchanged by treatment of isolated antigens with neuraminidase before proteolysis.

Despite the relative absence of discrete peptides from digests of the granulocyte antigen, successful proteolysis is confirmed by the reduced intensity of p110 bands in treated *v* control samples and a corresponding increase in counts present in low mol wt material at the gel front. All counts from the treated samples were transferred from the reaction vessels to the gel. Although comparable amounts of radioactive material were used in preparing all samples from the two cell types, some of those counts in the granulocyte samples reflect low mol wt species. Thus at least some of the differences in the bands between the two samples may be quantitative.

DISCUSSION

Our studies indicate that anti-CALLA MoAb identify antigens synthesized by both ALL cells and granulocytes

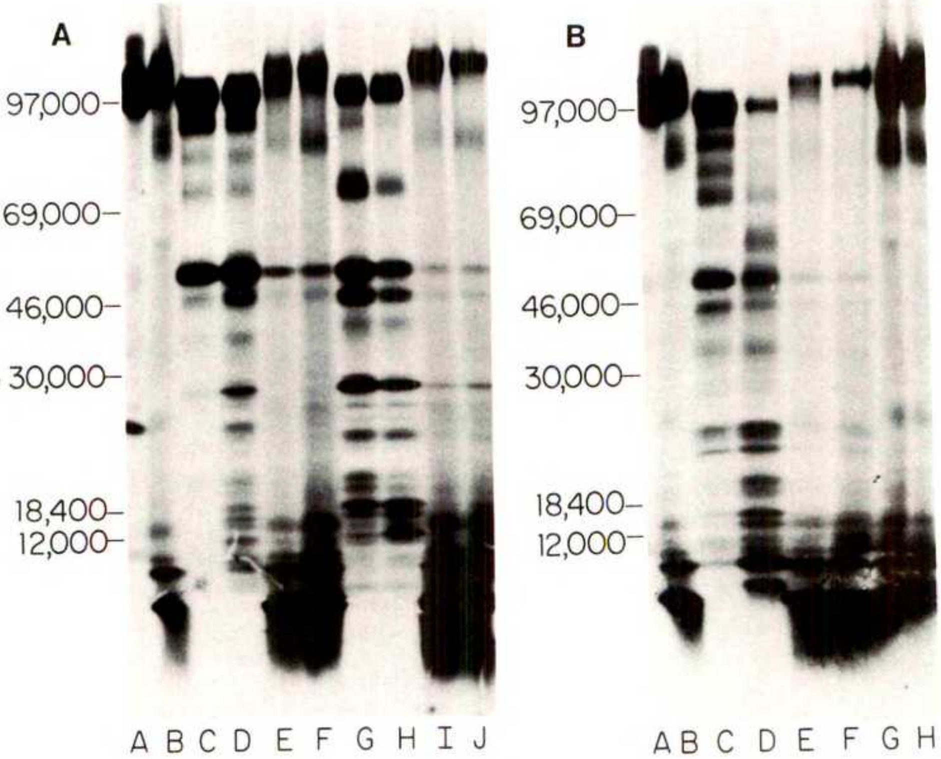

Fig 3. Proteolytic digests of iodinated antigens isolated from ALL cells (NALM-6) and human granulocytes by anti-CALLA-1 MoAb. Each antigen was treated with both 1 and 5 μg of each protease. Samples are as follows: Panel (A): A, sham-treated ALL antigen; B, sham-treated granulocyte antigen; C and D, ALL antigen treated with trypsin; E and F, granulocyte antigen treated with trypsin; G and H, ALL antigen treated with chymotrypsin; I and J, granulocyte antigen treated with chymotrypsin. Panel (B): A, sham-treated ALL antigen, B, sham-treated granulocyte antigen; C and D, ALL antigen treated with papain; E and F, granulocyte antigen treated with papain; G and H, sham-treated granulocyte antigen. Note the differences in mol wt between untreated antigens isolated from these two cell populations.

that have unique behavioral and chemical properties. Whereas the antigen on both normal and malignant lymphoid cells is readily modulated by specific antibody,[4-6] that on isolated granulocytes is not. The mol wt of the iodinated antigen on human granulocytes is higher than that on normal and malignant lymphoid cells and on the nonhematopoietic cells tested, indicating that previously observed differences were not due to individual variation.[9,10,12] In contrast, the mol wt of metabolically labeled antigen is lower in granulocytes than in lymphoid cells. Finally, proteolytic digests of the two antigens produce unique but overlapping banding patterns on SDS-PAGE.

Failure of the granulocyte antigen to modulate might reflect differences in either the integration of CALLA into the cell membrane of granulocytes and lymphocytes or the relative capacities of the surface structures of these two cell types to support antigenic modulation. Alternatively, the antigens detected on lymphoid cells and granulocytes may share an epitope(s) with one another but represent distinct chemical species. Because we have as yet no clear evidence that the five anti-CALLA MoAb used in our studies detect different antigenic determinants,[10] this latter possibility cannot be excluded.

Despite major differences in the antigens detected by anti-CALLA MoAb on ALL cells and granulocytes, it is still not known whether these antigens differ in their protein structures. The unique mol wt of these two antigens might well result from posttranslational modifications of a common peptide, such as the addition of carbohydrate residues.[17] Because differential glycosylation could modify both the sites of proteolysis and the mol wt of individual proteolytic fragments, the degree of homology of the peptide sequences of such antigens would be difficult to evaluate. Although analysis of the molecules from these two cell types in the total absence of glycosylation might clarify their relationship, production of such completely carbohydrate-free antigens is a difficult task. Amino terminal sequence analysis of the molecules on these two cell types might better address this issue.

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AIDS Retrovirus Antibodies in Hemophiliacs Treated With Factor VIII or Factor IX Concentrates, Cryoprecipitate, or Fresh Frozen Plasma: Prevalence, Seroconversion Rate, and Clinical Correlations

By Margaret V. Ragni, Gary E. Tegtmeier, Jay A. Levy, Lawrence S. Kaminsky, Jessica H. Lewis, Joel A. Spero, Franklin A. Bontempo, Cindy Handwerk-Leber, William L. Bayer, Daniel H. Zimmerman, and Judith A. Britz

Antibodies to the AIDS retrovirus, specifically to human T cell lymphotropic virus, type III, and AIDS-associated retrovirus, were detected with increasing prevalence in a population of 190 hemophiliacs from western Pennsylvania between 1981 and 1984: 7.7% in 1981, 20.0% in 1982, 45.5% in 1983, and 62.5% in 1984. The seropositive included approximately three fourths of those receiving factor VIII concentrate, nearly one third of those receiving factor IX concentrate, nearly one fifth of those receiving cryoprecipitate, and none of those receiving fresh frozen plasma. The seroconversion rate, determined on 43 seropositive hemophiliacs from this group who were serially sampled, was 0% in 1977, 4.7% in 1978, 4.9% in 1979, 2.6% in 1980, 10.5% in 1981, 52.9% in 1982, 87.5% in 1983, and 100% in 1984. Of 27 seropositive for three or more years (since 1982 or before), four (15%) have developed AIDS and seven (26%), diffuse lymphadenopathy (ARC); of 16 seropositive for less than three years, none has developed AIDS and three (19%) have developed ARC. The mean time from seroconversion to onset of ARC, 0.8 ± 0.2 years (SEM), was shorter ($P < .001$) than the time to onset of AIDS, 4.1 ± 0.6 years. These findings confirm the widespread presence of AIDS retrovirus and support the association of these retroviruses with the acquired immunodeficiency syndrome and related conditions.

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THREE GROUPS of investigators have isolated retroviruses from patients with the acquired immunodeficiency syndrome (AIDS) and AIDS-related syndromes. Termed lymphadenopathy-associated virus (LAV),[1] human T cell lymphotropic virus, type III (HTLV-III),[2] and AIDS-associated retrovirus (ARV),[3] these viruses appear to be nearly identical, with similar morphological, cytopathic, and molecular features.[1-5]

In hemophilia-related AIDS, transmission of the AIDS agent presumably occurs through exposure to blood products. Most hemophiliacs who have developed AIDS have been treated with factor VIII concentrate,[6] although the disease has been detected in several factor IX concentrate-treated hemophiliacs.[6] In addition, although AIDS has not yet occurred in hemophiliacs who have been treated with cryoprecipitate, two patients with von Willebrand's disease who were treated with cryoprecipitate have developed AIDS.[7] Correspondingly, AIDS retrovirus seropositivity has been detected primarily in those hemophiliacs receiving factor VIII concentrate,[8-12] to a lesser degree in those receiving factor IX concentrate, and in few, if any, receiving cryoprecipitate.[8-12]

This study evaluated the entire population of hemophiliacs from western Pennsylvania, exposed to factor VIII concentrate, factor IX concentrate, cryoprecipitate, or fresh frozen plasma, for the presence of antibodies to AIDS retroviruses, specifically antibodies to HTLV-III and ARV, between 1981 and 1984. This period coincided with the occurrence of AIDS in patients with hemophilia. Tests for antibodies to AIDS retrovirus isolates were used to determine whether any significant difference in antibody prevalence would be observed.

MATERIALS AND METHODS

Clinical population. Of the 190 hemophiliacs who constituted the entire hemophilic population in western Pennsylvania, 155 had hemophilia A and 35 had hemophilia B. Those with hemophilia A included 82 treated with factor VIII concentrate, 59 with cryoprecipitate, and 14 with factor IX concentrate because of the presence of an inhibitor to factor VIII. The last exposure to factor VIII concentrate in the latter group was before early 1976. Those with hemophilia B included 16 treated with factor IX concentrate and 19 with fresh frozen plasma. The mean age for those receiving factor VIII concentrate was 29.6 years, for factor IX concentrate 26.8 years, for cryoprecipitate 18.7 years, and for fresh frozen plasma 24.8 years.

Serum samples. Citrated plasma samples, obtained from all 190 hemophiliacs during routine clinical evaluation at the Hemophilia Center of Western Pennsylvania, were available for the assays described below. Only one blood sample per hemophiliac was tested in this study, specifically the first sample available beginning in 1984 and going back to 1981. In addition, 43 of the seropositive hemophiliacs on whom serial samples were available annually or biannually between 1977 and 1984 constituted the population base for determining the AIDS retrovirus seroconversion rate. This group, selected solely on the basis of their seropositivity and availability of serial samples, did not differ from the original 190 hemophiliacs in age, race, or blood product usage. Samples were frozen at −20 °C for several months to 6 years before the study.

Viral antibody tests. Antibodies (IgG) to HTLV-III were measured by an enzyme-linked immunosorbent assay (ELISA) technique (Electronucleonics, Inc, Columbia, Md), using an inactivated HTLV-III antigen from a previously established cell line.[11] Briefly, a 1:100 dilution of test plasma was added to a microassay-plate well that had been coated with inactivated virus antigen. After a 30-minute, 37 °C incubation, the unbound material was washed and aspirated. Goat antiserum to human IgG, both heavy and light chain

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specific, labeled with horseradish peroxidase, was then added and incubated for 30 minutes at 37 °C. Unbound material was washed and aspirated, and a chromogen (*o*-phenylenediamine dichloride, or OPD) was added. After a 10-minute, room temperature incubation, the reaction was stopped with 2N sulfuric acid, and the absorbance was read spectrophotometrically at 492 nm. Samples giving an optical density >0.100 in two separate assays were considered positive.

For seroconversion data only, antibodies to HTLV-III (IgG) were detected by an ELISA technique (Abbott Laboratories, Chicago), using beads coated with inactivated HTLV-III antigen from the Hg/HTLV-IIIB cell line. A 1:400 dilution of test plasma was added to each bead-containing microassay-plate well and was incubated 65 minutes at 40 °C. Unbound material was washed and aspirated. Goat antihuman IgG, conjugated to horseradish peroxidase, was added and incubated for 130 minutes at 40 °C. Unbound material was washed and aspirated, and the chromogen (OPD) was added and incubated for 30 minutes at room temperature. The reaction was stopped with 1N sulfuric acid, and absorbance was read at 492 nm. Samples giving an optical density greater than or equal to a cutoff value, determined daily by negative and positive controls, were considered positive.

Antibodies to ARV were measured, as previously described,[3] by indirect immunofluorescence (IFA), using goat antiserum to human IgG, an adult human T cell line, HUT-78, infected with ARV-2, and a 1:10 dilution of test plasma. Four of the original 190 samples were of insufficient quantity for ARV antibody testing. Samples that were HTLV-III (Electronucleonics) seropositive, ARV seronegative or HTLV-III (Electronucleonics) seronegative, ARV seropositive were retested at a 1:5 dilution of test plasma, and the latter result was used in this study. There was no significant difference in the results obtained by ELISA with the HTLV-III antigen and those obtained by IFA with the ARV-2 antigen.

For the Western blot procedures (Electronucleonics), purified HTLV-III virus was reduced and electrophoretically resolved by polyacrylamide gel electrophoresis, using 12.5% running and 4% stacking gels at 150 V, pH 8.8. The resolved protein was transferred to nitrocellulose electrophoretically at 36 V for four hours, blocked with 20% goat serum in phosphate-buffered saline (PBS)/0.05% Tween (J.T. Baker Chemical Co, Phillipsburg, NJ), and cut into 0.5-cm strips. These strips were loaded with a 1:200 dilution of test plasma, incubated for two hours at room temperature, washed, and then incubated with a 1:2,000 dilution of goat antihuman IgG (heavy and light chain) conjugated to horseradish peroxidase. After serial washings, the substrate chloro-naphthol was added, and the strips were developed, blotted on bibulous paper, and dried. The test was considered positive if either the viral p24 or gp41 proteins were present.

Table 1. Prevalence of AIDS Retrovirus Antibodies in a Population of 190 Hemophiliacs

	AIDS Retrovirus Antibody	
	Number	Percentage
Prevalence by year sampled		
1981	2/26 (2/26)	7.7 (7.7)
1982	8/40 (6/40)	20.0 (15.0)
1983	20/44 (16/42)	45.5 (38.1)
1984	50/80 (54/78)	62.5 (69.2)
Total	80/190 (78/186)	42.1 (41.9)
Prevalence by blood product exposure		
F VIII	61/82 (60/81)	74.4 (74.1)
F IX	9/30 (10/30)	30.0 (33.3)
Cryo	10/59 (8/57)	17.0 (14.0)
FFP	0/19 (0/18)	0 (0.0)
Total	80/190 (78/186)	42.1 (41.9)

The data presented is the number (or percentage) seropositive over the total number tested for HTLV-III antibody, with similar data based on ARV antibody in parentheses. Only one blood sample per hemophiliac was used in this study—the first sample available from 1984 back to 1981. F VIII, factor VIII concentrate; F IX, factor IX concentrate; CRYO, cryoprecipitate; FFP, fresh frozen plasma.

Statistical analysis was performed by Student's *t* test.

RESULTS

The progressive yearly increase in AIDS retrovirus seropositivity in this group of hemophiliacs between 1981 and 1984 is shown in Table 1, with an overall prevalence for these years of 42.1% (80/190), respectively. The 80 seropositive included 74.4% of those receiving factor VIII concentrate, 30.0% of those receiving factor IX concentrate, 17.0% of those receiving cryoprecipitate, and 0% of those receiving fresh frozen plasma, respectively. The prevalence of seropositivity in factor IX concentrate-treated patients with hemophilia B was similar to that of factor IX concentrate-treated patients with hemophilia A and an inhibitor: of nine seropositive, five had hemophilia B and four had hemophilia A with an inhibitor.

Forty-three seropositive hemophiliacs from the group, on whom serially samples were available between 1977 and 1984, were further studied. None were seropositive in 1977 and two were seropositive in 1978. An increase in seroconversion rate occurred after 1980, with the most marked increase appearing in 1982 (Table 2). Seropositivity persisted in all individuals on repeat testing at later dates, and none reverted back to seronegative after becoming seropositive. When the date of first seroconversion was looked at in each blood product treatment group (Table 3), the first of 25 factor VIII concentrate-treated hemophiliacs seroconverted in 1978, the first of eight factor IX concentrate-treated hemophiliacs seroconverted in 1978, and the first of ten cryoprecipitate-treated hemophiliacs seroconverted in 1981. (The latter is a locally prepared single-donor product.) The eight factor IX concentrate-treated hemophiliacs included four factor IX-deficient individuals, three of whom seroconverted in 1983

Table 2. AIDS Retrovirus Seroconversion Rate

Year	No. Becoming Seropositive/ No. Remaining Seronegative	Percentage
1977	0/43	0.0
1978	2/43	4.7
1979	2/41	4.9
1980	1/39	2.6
1981	4/38	10.5
1982	18/34	52.9
1983	14/16	87.5
1984	2/2	100.0

The determination of seroconversion rate was based on serial samples on 43 AIDS retrovirus seropositive hemophiliacs between 1977 and 1984. Seroconversion rate was expressed as a percentage and defined for each year as the number becoming seropositive (seroconverting) in a given year over the number remaining seronegative that year.

Table 3. AIDS Retrovirus Seroconversion in 43 Seropositive Hemophiliacs

	1977	1978	1979	1980	1981	1982	1983	1984
F VIII*								
1†	−	−	−	−	+	+		+
2†	−	−	−	+	+	+	+	+
3†	−	+	+	+	+	+		+
4†	−	−	−	−	−	+	+	+
5‡		−	+	+	+		+	+
6‡	−	−	−	−	−	+		+
7‡	−	−	−	−	−	+		+
8‡	−	−	−	−	+	+		+
9‡	−	−	−	−	−	+		+
10‡	−	−	−	−	−	+		+
11‡	−	−	−	−	−	−	+	+
12‡		−	+	+	+	+		+
13	−	−	−	−	−	+	+	+
14	−	−	−	−	−	+	+	+
15	−	−	−	−		+	+	+
16	−	−	−	−	−	−	+	+
17			−			−	+	+
18	−	−		−	+	+	+	+
19		−	−		−	+		+
20	−	−	−	−	−	+	+	+
21		−	−	−	−	+	+	+
22	−	−	−	−	−		+	+
23	−	−	−		−	+	+	+
24		−	−		−	+	+	+
25	−	−		−	−	−	+	+
F IX*								
1		−	−	−		−	+	+
2	−	−	−	−	−	−	−	+
3‡	−	−	−	−	−	−	+	+
4		−	−	−	−		+	
5	−	−	−	−	−	+	+	+
6		+	+	+		+		+
7	−	−	−	−	−	−	+	+
8‡	−	−	−	−	−	−	+	+
Cryo*								
1	−	−	−		−	−		+
2		−	−	−	−	−	+	+
3					−	+		+
4		−	−		−	−	+	+
5	−	−	−	−	−	+	+	
6					−	−	+	+
7	−	−	−	−	−	−	+	+
8					−	+		+
9			−	−	−	+		
10	−	−	−		+			

*F VIII refers to hemophiliacs treated with factor VIII concentrate, F IX refers to those treated with factor IX concentrate, and cryo refers to those treated with cryoprecipitate.

†Patient developed AIDS.

‡Patient developed diffuse lymphadenopathy (ARC).

and the fourth of whom seroconverted in 1984; the other four factor IX concentrate-treated hemophiliacs included four factor VIII-deficient individuals with an inhibitor to factor VIII whose last exposure to factor VIII was before early 1976, one of whom seroconverted in 1978, one in 1982, and two in 1983.

From this group of 43 sequentially sampled hemophiliacs, four (9%) developed AIDS and ten (23%) developed diffuse lymphadenopathy (ARC). Of the 27 who have been seropositive for three or more years (1982 or earlier seroconversion), four (15%) have developed AIDS and seven (20%) have developed diffuse lymphadenopathy (Table 3). In addition, one (4%) developed immune thrombocytopenia (also had AIDS). Of the 16 who have been seropositive for less than three years (1983 or later seroconversion), none has developed AIDS and three (19%) have developed ARC. The mean time from seroconversion to onset of ARC in all ten with ARC was 0.8 ± 0.2 years (SEM) (range, 0 to 2) which was significantly shorter ($P < .001$) than the time to onset of AIDS in all four with AIDS, 4.0 ± 0.6 years (range, 3 to 6).

The total yearly blood product usage (units per year) in patient sampled in 1984 (Table 4) revealed that a significantly greater amount of factor VIII concentrate was used in seropositive hemophiliacs than in seronegative hemophiliacs. The factor IX concentrate usage appeared to be twice as high in seropositive hemophiliacs as in seronegative hemophiliacs, but this difference was not significant. There appeared to be no difference in cryoprecipitate usage between seropositive and seronegative hemophiliacs. Because of small numbers and incomplete data, comparisons could not be made for 1981 through 1983, except for factor VIII concentrate usage in 1983, which was significantly greater ($P < .005$) (data not shown) in seropositive hemophiliacs compared with seronegative hemophiliacs.

DISCUSSION

The increasing prevalence of antibodies to AIDS retrovirus in this group of hemophiliacs between 1981 and 1984 parallels the occurrence of AIDS in the hemophiliac population.[6] Further, the strong correlation between the presence of antibodies to HTLV-III and ARV in these patients measured by different assays is consistent with data showing that HTLV-III and ARV (as well as LAV) are variants of the same virus.[4,5] The fact that all hemophiliacs with AIDS or AIDS-related illness in this study were AIDS retrovirus (both HTLV-III and ARV) seropositive supports the close association of these retroviruses with AIDS and AIDS-related conditions. Detection of AIDS retrovirus antibodies (HTLV-III or ARV) in the majority of western Pennsylva-

Table 4. Blood Product Usage and AIDS Retrovirus Antibody Status in 1984

Blood Product	Antibody (+)	Antibody (−)	*P*
F VIII concentrate	66,637 ± 13,086 (n = 41)	18,603 ± 7,354 (n = 6)	<.005
F IX concentrate	58,053 ± 12,951 (n = 4)	27,310 ± 7,101 (n = 5)	NS
Cryoprecipitate	19,000 ± 6,223 (n = 3)	19,925 ± 5,476 (n = 8)	NS
Fresh frozen plasma	— (n = 0)	7,585 ± 4,819 (n = 2)	—

Data are based on blood product usage between September 1983 and August 1984. Figures in parentheses are the number on whom data were available. Data presented are based on HTLV-III antibody positive and negative hemophiliacs; corresponding data for ARV antibody positive and negative were not significantly different. Results are expressed as the mean ± standard error (SEM).

nia hemophiliacs, similar to data reported in San Francisco homosexuals[3] and hemophiliacs[12] and in Australian bisexual and homosexual males,[13] demonstrates that exposure to the AIDS retrovirus is widespread geographically. Moreover, the AIDS virus appears to have been present here at least since 1978, one year earlier than that found in a smaller study[14] by Eyster et al, but still consistent with the occurrence of the earliest cases of AIDS in the United States in 1978 and 1979.

The predominance of AIDS retrovirus seropositivity in factor VIII concentrate-treated hemophiliacs parallels the higher incidence of AIDS in hemophiliacs treated with this blood product. The markedly lower prevalence of antibodies in hemophiliacs exposed to factor IX concentrate is consistent with the infrequent occurrence of AIDS in this group.[6] The presence of such antibodies in cryoprecipitate-treated hemophiliacs, a group in which AIDS has not yet occurred, further supports the recommendation of the use of heat-treated factor VIII concentrate to prevent transmission of potentially infectious retroviruses.[15,16]

The marked increase in seroconversion that occurred in 1982 is in agreement with a similar study of hemophiliacs treated only with factor VIII concentrate.[14] The time of occurrence and the percentage of virus-exposed individuals developing disease are a noteworthy observation. They offer some indication of the risk of pathogenic consequences after exposure to the virus in hemophiliacs and mirror similar observations with other AIDS risk groups.[17] Further, these data suggest that a large number of hemophiliacs were exposed through their clotting factor treatment to infectious viruses and not just viral antigens. The short time between the date of seroconversion and the development of diffuse adenopathy in ten hemophiliacs supports the view that this clinical sign can be an early manifestation of AIDS retrovirus infection or exposure. The reason for the longer time between seroconversion and development of AIDS in four hemophiliacs is not clear but probably reflects the indolent disease course that eventually leads to widespread compromise of the immune system by AIDS retrovirus infection.

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In Situ Quantitation of Lymph Node Helper, Suppressor, and Cytotoxic T Cell Subsets in AIDS

By Gary S. Wood, Bruce F. Burns, Ronald F. Dorfman, and Roger A. Warnke

We have used the novel monoclonal antibodies 9.3 and anti-Leu-8 in conjunction with other T cell markers to quantify T cell subpopulations in the paracortex, mantle, and germinal center compartments of frozen sections of lymph nodes from seven homosexual men with acquired immunodeficiency syndrome (AIDS) and five heterosexual controls. Antibody 9.3 allows dissection of the Leu-2$^+$ cytotoxic/suppressor subset (T_{cs}) into 9.3$^+$ cytotoxic cells (T_c) and 9.3$^-$ suppressor cells (T_s). Anti-Leu-8 allows dissection of the Leu-3$^+$ helper subset (T_H) into functionally distinct subpopulations. The data indicate that the T cells in patients with AIDS exhibit normal antigen expression but altered subset ratios. In this series, the data suggested that the reversal of the paracortical T_H-T_{cs} ratio was due to an increase in T_s with a concomitant decrease in T_H and T_c. These changes were also reflected in a reversal of the normal paracortical T_c-T_s ratio (3.0) to <1.0. Furthermore, the data suggested a marked decrease in paracortical Leu-3$^+$8$^+$$T_H$, which are known to have inducer function in cellular immune reactions and exert feedback inhibition of immunoglobulin production through a suppressor T cell intermediary. In contrast, there was preservation of the Leu-3$^+$8$^-$$T_H$ population within the germinal center. This T cell subset is known to help B cell differentiation. This microenvironmentally specific constellation of T cell subset alterations within lymph nodes may in part explain several of the immunologic findings associated with AIDS.

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DURING THE PAST FEW YEARS, several studies have documented quantitative and qualitative abnormalities of peripheral blood T cells obtained from homosexual men with acquired immunodeficiency syndrome (AIDS).[1-13] These abnormalities include decreased total circulating T cells, a decreased helper-cytotoxic/suppressor ratio, reduced response to mitogens, and diminished in vitro helper activity. Similar peripheral blood abnormalities have been identified in patients from other groups at risk for AIDS.[4,8-11,14-20] Related immunohistologic studies of T cells within lymph nodes have documented changes within the paracortical T cell domain that parallel those noted previously in the blood.[9,21-23]

These immunophenotypic studies of blood and lymphoid tissue have generally used a relatively small panel of monoclonal antibodies, including anti-Leu-3(T4) and anti-Leu-2(T8). Consequently, it has been difficult to determine whether T cell populations in AIDS exhibit normal or abnormal patterns of antigen expression, as often occurs in T cell neoplasia.[24-26] Furthermore, although commonly referred to as the helper-suppressor ratio, the Leu-3(T4)-Leu-2(T8) ratio is actually a comparison of two heterogeneous T cell subsets.[27,28] The availability of two additional monoclonal antibodies, anti-Leu-8[27] and 9.3,[28] has made it possible to further dissect these subsets. The Leu-3(T4)$^+$ subset contains Leu-8$^-$ helper T cells (T_H) that help B cell differentiation and Leu-8$^+$ T_H primarily concerned with other types of inducer function.[27] The Leu-2(T8)$^+$ subset contains 9.3$^+$ cytotoxic T cells (T_c) as well as 9.3$^-$ suppressor T cells (T_S).[28]

Recent studies of peripheral blood T cells in homosexual men with the syndrome of persistent generalized lymphadenopathy (PGL) have documented decreased T4$^+$Leu-8$^+$ cells and increased T8$^+$Leu-8$^-$ cells.[18] In the present study, we have used immunoperoxidase techniques and a large panel of anti-T cell monoclonal antibodies, including anti-Leu-8 and 9.3, to characterize and quantitate T cell subsets in situ in lymph nodes from homosexual men with AIDS and heterosexual controls.

MATERIALS AND METHODS

Patients and controls. Lymph node biopsies were obtained from seven homosexual men with AIDS and five heterosexual controls. The selection of patients was based on the availability of frozen tissue for immunohistologic studies. The average patient age was 32 years (range, 25 to 49). One patient died of cytomegalovirus/*Pneumocystis carinii* pneumonia (CMV/PCP). The remaining six patients were alive with cutaneous or nodal Kaposi's sarcoma. Generalized lymphadenopathy was present in all patients except one with Kaposi's sarcoma. The selection of heterosexual controls was determined by diagnosis of nonspecific reactive follicular hyperplasia in lymph nodes, the lack of generalized lymphadenopathy, and the availability of frozen tissue. The average control age was 30 years (range, 17 to 41).

Lymph nodes from patients with Kaposi's sarcoma demonstrated reactive follicular hyperplasia, sometimes with partial effacement of follicular mantle zones. In those lymph nodes that were partially involved by Kaposi's sarcoma, there was less prominent follicular hyperplasia. Regressively transformed germinal centers were also observed. Lymph nodes from the patient with CMV/PCP exhibited a lymphocyte depletion pattern manifested by decreased paracortical lymphocytes, fibrosis, and absent B cell follicles. The histopathology of some of these lymph node biopsies has been detailed elsewhere.[29]

Immunohistology. All lymph nodes were obtained at surgical biopsy except for those from the patient with CMV/PCP. The latter were obtained at autopsy within a few hours of death. Representative portions were cryopreserved, sectioned, and stained with a three-stage monoclonal antibody-biotin-avidin immunoperoxidase technique, as described previously.[30] The panel of monoclonal antibodies that was used is listed in Table 1, along with predominant specificities and sources.

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Table 1. Monoclonal Antibodies

Antibody (Antigen)	Predominant Specificity
Anti–Leu-1 (CD5)	T cells, thymocytes
Anti–Leu-2 (CD8)	Cytotoxic/suppressor T cells, thymocytes
Anti–Leu-3 (CD4)	Helper T cells, thymocytes, histiocytes
Anti–Leu-4 (CD3)	T cells, thymocytes
Anti–Leu-5 (CD2)	T cells, thymocytes
Anti–Leu-6 (CD1)	Thymocytes, Langerhans cells
Anti–Leu-8	Blood: 75% ± 10% Leu-3$^+$ T cells; 60% ± 10% Leu-2$^+$ T cells; majority of B cells, NK cells, monocytes, and granulocytes
9.3	Blood: Helper T cells, cytotoxic T cells
OKT9 (T9, transferrin receptor)	Thymocytes, several other actively proliferating cell types, including germinal center cells
OKT10 (T10)	Thymocytes, plasma cells
L203 (HLA-DR)	B cells, histiocytes, activated T cells

Antibodies were obtained from the following sources: Anti–Leu-1, -2, -3, -4, -5, -6, and -8 from Becton Dickinson, Mountain View, Calif., 9.3 from John Hansen, Seattle, courtesy of Edgar Engleman, Stanford University; OKT9 and OKT10 from Ortho Pharmaceuticals, Raritan, NJ; L203 from Ronald Levy, Stanford University.

The peripheral blood reactivity of anti–Leu-8 and 9.3 is detailed elsewhere[27,28] but will be reviewed here briefly (Table 2). Anti–Leu-8 stains 75% ± 10% of Leu-3$^+$ cells, 60% ± 10% of Leu-2$^+$ cells, and the majority of B cells, natural killer cells, monocytes, and granulocytes. Its reactivity has been reported to be similar to that of another monoclonal antibody, anti-TQ1,[18] although these two antibodies do not cross-block.[27] Antibody 9.3 stains virtually all Leu-3$^+$ cells and approximately 50% of Leu-2$^+$ cells in peripheral blood. Leu-2$^+$ 9.3$^+$ T cells have cytotoxic function, whereas Leu-2$^+$ 9.3$^-$ T cells have suppressor function.[28] Within the peripheral blood, 9.3 staining appears to be restricted to T cells.[28]

Table 2. Dissection of T Cell Phenotypes With Monoclonal Antibodies

	T Cell Phenotype		
Antibody	T_H	T_C	T_S
Leu-2	−	+	+
Leu-3	+	−	−
Leu-4	+	+	+
9.3	+	+	−
9.3 − Leu-3	−	+	−
Leu − 4-9.3	−	−	+
Leu-8	+/−	+/−	+/−

In Situ *quantitation of T cell subsets.* T cell subsets were quantitated within the major T and B cell compartments of the lymph node: the paracortex (T cell domain), the mantle zone (B cell domain), and the germinal center (B cell domain) (Table 3). Semiserial sections were scanned at lower power to select two areas representative of each compartment. All cell counts were obtained from these same two areas. A square, cross-hatched grid mounted in an American Optical (Buffalo) 15× microscope eyepiece was then used to count cells under the 40× objective. At this magnification, the grid measured 0.25 mm on each side. The cells within the grid area that stained with anti–Leu-2, -3, -4, -8, or 9.3 were counted. The two values generated for each antibody were then averaged to give the mean value for the compartment as a whole. Histiocytes, which were variably Leu-3$^+$,[31] were excluded from Leu-3$^+$ T cell counts.

Based on the phenotypic and functional data reviewed above and in Tables 1 and 2, T cell subsets were defined as follows: T cells (Leu-4$^+$), helper T cells (T_H) (Leu-3$^+$), cytotoxic/suppressor T cells (T_{cs}) (Leu-2$^+$), cytotoxic T cells (T_c) (9.3$^+$ minus Leu-3$^+$), and suppressor T cells (T_s) (Leu-4$^+$ minus 9.3$^+$). Various T cell subset ratios were then calculated based on these values. The data in Table 3 represent the means for each T cell subset or ratio calculated from the seven homosexual men with AIDS and the five controls. Quantitation of paracortical T cell subsets as percentages of the combined

Table 3. Quantitation of T Cell Subsets in AIDS *v* Controls

	Paracortex		Mantle		Germinal Center	
T Cell Subset or Ratio	AIDS (n = 7)	Controls (n = 5)	AIDS (n = 5)	Controls (n = 5)	AIDS (n = 6)	Controls (n = 5)
Total T	382*	849	107	148	72	58
	(174–628)	(746–928)	(50–140)	(85–178)	(51–94)	(44–94)
T_H	124*	678	64	104	49	55
	(26–243)	(550–756)	(36–89)	(46–160)	(36–63)	(36–97)
T_{cs}	242	186	50	37	25*	4
	(140–440)	(128–291)	(26–90)	(10–58)	(14–36)	(1–12)
T_c	38*	131	12	20	5	3
	(1–96)	(72–233)	(2–43)	(1–36)	(1–8)	(1–4)
T_s	211*	44	33	12	19*	3
	(120–357)	(30–69)	(10–50)	(5–17)	(9–31)	(1–10)
T_H-T_{cs}	0.54*	4.06	1.58	4.25	2.10*	33.43
	(0.17–0.79)	(2.15–5.91)	(0.58–3.30)	(2.07–7.50)	(1.39–2.93)	(3.00–56.00)
T_H-T_s	0.63*	16.85	2.48*	10.42	2.83*	34.42
	(0.17–0.86)	(9.07–24.40)	(1.16–4.23)	(7.87–15.00)	(1.87–4.44)	(3.60–97.00)
T_c-T_s	0.19*	2.96	0.37	1.67	0.27	1.54
	(0.01–0.43)	(1.64–4.16)	(0.05–0.95)	(0.06–3.00)	(0.06–0.53)	(0.20–4.00)

The values shown are the means of the absolute number of cells per grid area, with the range in parentheses.

*$P \leq .01$ relative to controls; $P > .05$ for all other values.

Table 4. Quantitation of Paracortical Leu-8⁺ Cells and Non-T Cells in AIDS v Controls

	Leu-8⁺ Cells	T015⁺ (B) Cells	Leu-M3⁺/ 63D3⁺ Cells (Histiocytes)	Leu-7⁺ (NK) Cells
Controls	866	61	14	3
(n = 5)	(814-934)	(22-110)	(5-20)	(0-6)
AIDS	262*	97	42*	9*
(n = 7)	(70-644)	(62-156)	(18-79)	(5-14)

The values shown are the means of the absolute number of cells per grid area, with the range in parentheses.

*$P \leq .01$ relative to controls.

paracortical T cell, B cell, histiocyte, and NK (natural killer/killer) cell population also required quantitation of the absolute number of paracortical T015⁺ B cells,[32] Leu-M3⁺ or 63D3⁺ histiocytes,[33,34] Leu-7⁺ NK cells,[35] and Leu-8⁺ cells (Table 4). These additional data concerning non-T cell subsets are presented in detail elsewhere.[36] Statistical significance was assessed with the two-tailed Wilcoxon two-sample rank test (Mann-Whitney test).

The internal consistency of the quantitation technique was assessed by comparing the sum of Leu-2⁺ and Leu-3⁺ cells v Leu-4⁺ cells in all three tissue compartments in both patients and controls. As shown in Table 5, there was good correlation between these two values in each instance. Furthermore, the number of T_c (9.3⁺ minus Leu-3⁺) was always less than the number of T_{cs} (Leu-2⁺). Similarly, T_s (Leu-4⁺ minus 9.3⁺) was always less than T_{cs} (Leu-2⁺).

Estimation of Leu-3⁺8⁺ paracortical cells. A minimum/maximum analysis was performed in order to estimate the difference in the number of Leu-3⁺8⁺ paracortical cells in patients v controls (Table 5). The maximum possible absolute number of Leu-3⁺8⁺ cells was either the Leu-3⁺ cell total or the Leu-8⁺ cell total—whichever was less. The minimum possible absolute number of Leu-3⁺8⁺ cells was either 0 or the total absolute number of cells in all other subsets (T_{cs}, B cell, histiocyte, and NK) subtracted from the Leu-8⁺ cell total—whichever was greater. The absolute numbers of cells in each subset from each patient and control were used for these calculations (Tables 3 and 4). The absolute numbers of Leu-3⁺8⁺ cells were also expressed as percentages relative to total Leu-3⁺ cells.

RESULTS

Controls. The paracortical T_H-T_{cs} (Leu-3⁺-Leu-2⁺) ratio averaged 4.06 in controls (Table 3). Further dissection of the Leu-2⁺ T_{cs} subset with antibody 9.3 indicated that cells of T_c phenotype were the predominant Leu-2⁺ subpopulation with a T_c-T_s ratio averaging 2.96. As such, the calculated T_H-T_s ratio averaged 16.85. This was considerably higher than the Leu-3⁺-Leu-2⁺ ratio (4.06), which, although often referred to as the helper-suppressor T cell ratio, actually represents the helper-cytotoxic/suppressor T cell ratio. The mean absolute values for control paracortical T_H, T_c, and T_s (Table 3) are shown in Fig 1.

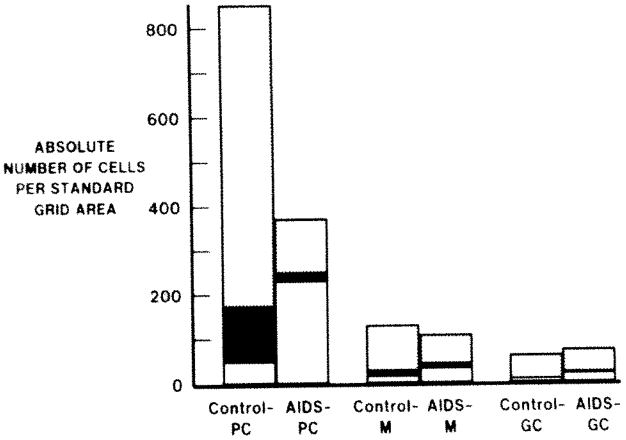

Fig 1. Alterations of T cell subsets in AIDS v controls. The histogram is based on the mean values for T_H (gray), T_c (black), and T_s (white) as detailed in Table 3. Within the paracortex (PC) in AIDS, there was a marked reduction in total T cells, T_H, and T_c, while T_s were increased ($P \leq .01$). No statistically significant quantitative alterations were noted within the mantle (M); however, within the germinal center (GC), T_s were increased ($P \leq .01$).

Quantitation of 9.3⁺ cells and comparison to Leu-4⁺ cells revealed that 9.3 stained slightly fewer cells than anti–Leu-4 within the paracortex (mean, 95%), mantle (mean, 92%), and germinal center (mean, 94%). Both antibodies had a similar distribution pattern, suggesting that 9.3 is restricted to cells of T lineage. In contrast, anti–Leu-8 stained the vast majority of cells within both the paracortex and the mantle, which contain a predominance of T cells and B cells, respectively. Furthermore, Leu-8⁺ cells were virtually absent within the germinal center. Representative immunohistologic staining of control lymph nodes is shown in Fig 2.

Leu-8⁺ cells, B cells, histiocytes, and NK cells were quantitated within the paracortex (Table 4). The immunohistology of the non-T cells is presented in detail elsewhere.[36] In conjunction with the paracortical values listed in Table 3, these data allowed calculation of the percentages of various T cell subsets relative to the total number of paracortical T cells, B cells, histiocytes, and NK cells (Fig 3). These data indicated that within the paracortex, the majority of total T cells, T_H, T_{cs}, and T_c were Leu-8⁺, since Leu-8⁻ cells constituted only 6% of the paracortical combined T cell, B cell, histiocyte, and NK cell population, whereas these T cell groups constituted 92%, 74%, 20%, and 14%, respectively. In

Table 5. Internal Consistency of Quantitation Technique: Leu-2⁺ + Leu-3⁺ v Leu-4⁺

	Paracortex (Controls, n = 5; AIDS, n = 7)		Mantle (Controls, n = 5; AIDS, n = 5)		Germinal Center (Controls, n = 5; AIDS, n = 6)	
	Leu-2⁺ + Leu-3⁺	Leu-4⁺	Leu-2⁺ + Leu-3⁺	Leu-4⁺	Leu-2⁺ + Leu-3⁺	Leu-4⁺
Controls	864	849	154	148	59	58
	(746-917)	(746-982)	(85-183)	(85-178)	(44-99)	(44-94)
AIDS	375	382	114	107	74	72
	(176-615)	(174-628)	(62-142)	(50-140)	(55-94)	(51-94)

The values shown are the means of the absolute number of cells per grid area, with the range in parentheses.

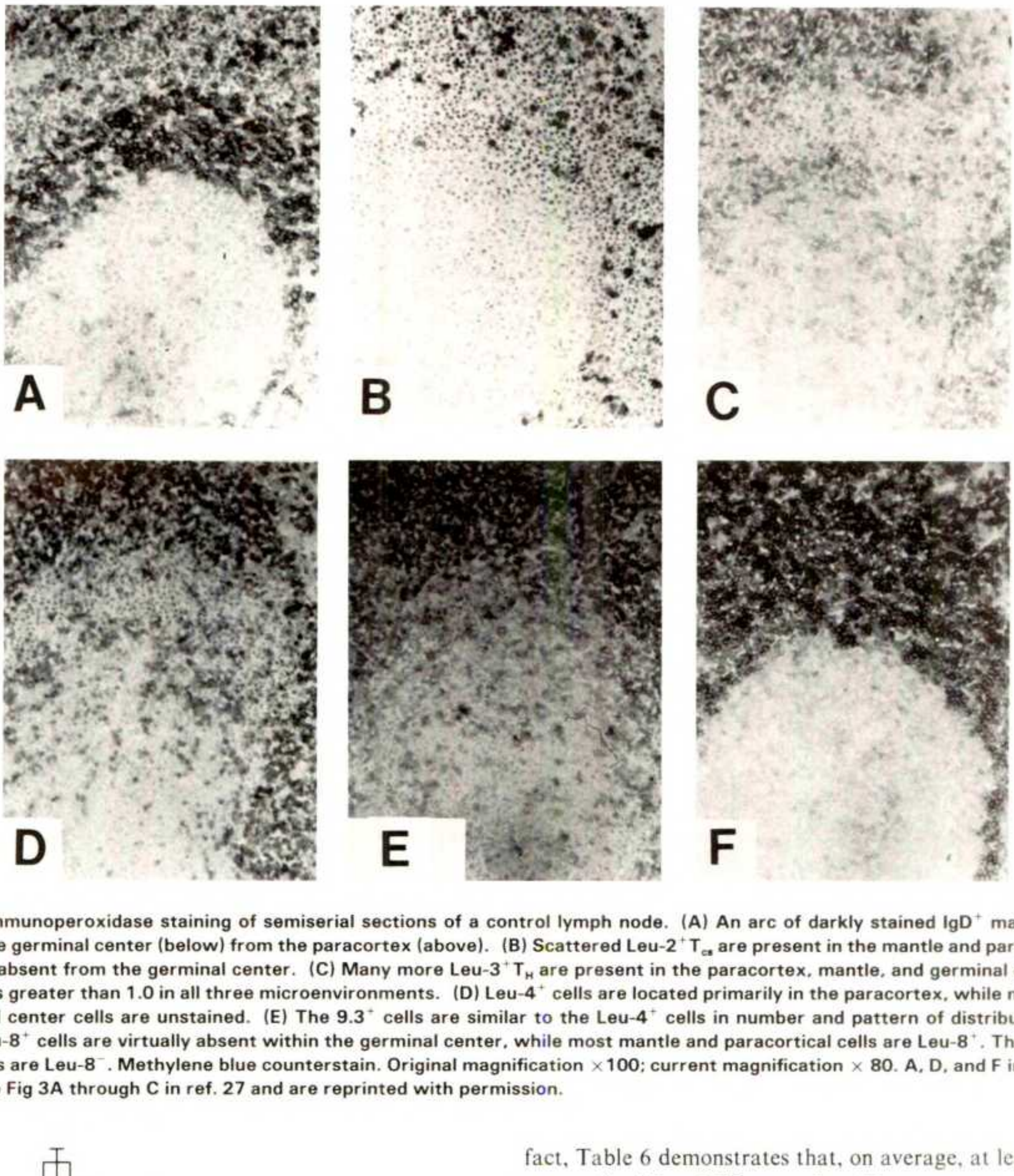

Fig 2. Immunoperoxidase staining of semiserial sections of a control lymph node. (A) An arc of darkly stained IgD^+ mantle B cells separates the germinal center (below) from the paracortex (above). (B) Scattered Leu-2^+ T_{cs} are present in the mantle and paracortex but are virtually absent from the germinal center. (C) Many more Leu-3^+ T_H are present in the paracortex, mantle, and germinal center. The T_H-T_{cs} ratio is greater than 1.0 in all three microenvironments. (D) Leu-4^+ cells are located primarily in the paracortex, while most mantle and germinal center cells are unstained. (E) The 9.3^+ cells are similar to the Leu-4^+ cells in number and pattern of distribution. (F) In contrast, Leu-8^+ cells are virtually absent within the germinal center, while most mantle and paracortical cells are Leu-8^+. Thus germinal center T cells are Leu-8^-. Methylene blue counterstain. Original magnification ×100; current magnification × 80. A, D, and F in this figure are similar to Fig 3A through C in ref. 27 and are reprinted with permission.

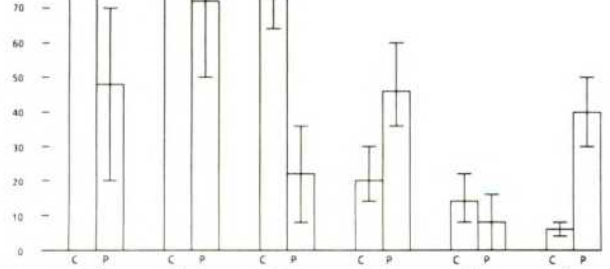

Fig 3. Paracortical Leu-8^+ and T cell subsets: relative alterations in AIDS *v* controls. Calculations are based on the absolute numbers of paracortical T, T_H, T_{cs}, T_c, T_s, Leu-8^+ cells, B cells, histiocytes, and NK cells as listed in Tables 3 and 4. Percentages are expressed relative to total T cells, B cells, histiocytes, and NK cells. Each bar represents the mean value with the range indicated by the T bar extensions. $P \leq .01$ relative to controls for each subset diagrammed except T_c. C, controls; P, patients.

fact, Table 6 demonstrates that, on average, at least 90% of paracortical Leu-3^+ T_H were Leu-8^+.

In addition to Leu-4, T cells within all three compartments also stained for other mature T cell antigens (Leu-1 and Leu-5), lacked immature T cell antigens (Leu-6, OKT9, OKT10), and were generally HLA-DR^-.

Homosexual men with AIDS. When compared with the control group (Tables 3 and 4), the homosexual men with AIDS exhibited several statistically significant differences ($P < .01$): (1) total T cells, T_H, and T_c were decreased in the paracortex; (2) T_s were increased in the paracortex and germinal center; (3) although the T_H-T_s ratio was decreased in all three compartments, only the paracortex showed a reversal of this ratio of <1.0; (4) the T_c-T_s ratio was reversed to <1.0 in the paracortex: (5) Leu-8^+ cells were decreased in the paracortex; (6) histiocytes and NK cells were increased in the paracortex. Virtually all germinal center cells were Leu-8^-, and there was no significant change in the number of Leu-3^+ T_H in this compartment. Alterations in the mean absolute number of T_H, T_c, and T_s are shown in Fig 1.

Table 6. Quantitation of Paracortical Leu-3^+8^+ Cells

				Leu-3^+8^+: Minimum* and Maximum† Limits	
	Leu-3^+	Leu-8^+	Leu-2^+, Leu-7^+, Leu-M3^+, and T015^+	Absolute Number	Percentage Relative to Leu-3^+ Cells
Controls	678	866	264	603 – 678	90 – 100
(n = 5)	(550–756)	(814–934)	(178–365)	(533–697) (550–756)	(81–99) (100)
AIDS	124‡	262‡	390	5‡ – 124‡	2‡ – 100
(n = 7)	(26–243)	(70–466)	(266–552)	(0–36) (26–243)	(0–15) (100)

The values shown are the means of the absolute number of cells per grid area (except where labeled as percentages) with the range in parentheses.

*Zero or [Leu-8^+-(Leu-2^+, Leu-7^+, Leu-M3^+, and T015^+)], whichever was greater. See Materials and Methods for further details.

†Leu-3^+ or Leu-8^+, whichever was less. See Materials and Methods for further details.

‡$P \leq .01$ relative to controls.

Representative immunohistologic staining of AIDS lymph nodes is shown in Figs 4 and 5.

The marked decrease in Leu-8^+ paracortical cells from 94% in controls to 48% in AIDS patients (Fig 3) was further explored for the Leu-3^+ T cell subset. Table 6 demonstrates that the absolute number of paracortical Leu-3^+8^+ T_H was severely reduced in AIDS *v* controls. Although these data did not reveal the exact size of the reduction, they did allow calculation of the limits of this decrease. The maximum possible decrease was the control maximum (678) minus the AIDS minimum (5), which equals 673, or 99% of the control maximum. The minimum possible decrease was the control minimum (603) minus the AIDS maximum (124), which equals 479, or 79% of the control minimum. Thus the actual reduction of Leu-3^+8^+ T_H in AIDS fell somewhere between the limits of 479 and 673 cells per grid area, which represents a decrease of at least 79%. Because a similar analysis of the paracortical Leu-3^+8^- subset was consistent with either an increase or a decrease in AIDS *v* controls, it was not possible to determine how this subset was quantitatively altered in the paracortex in AIDS. However, because germinal centers were essentially devoid of Leu-8^+ cells, it was apparent that the increased T_s and the unchanged T_H within the germinal center belonged to the Leu-8^- subset.

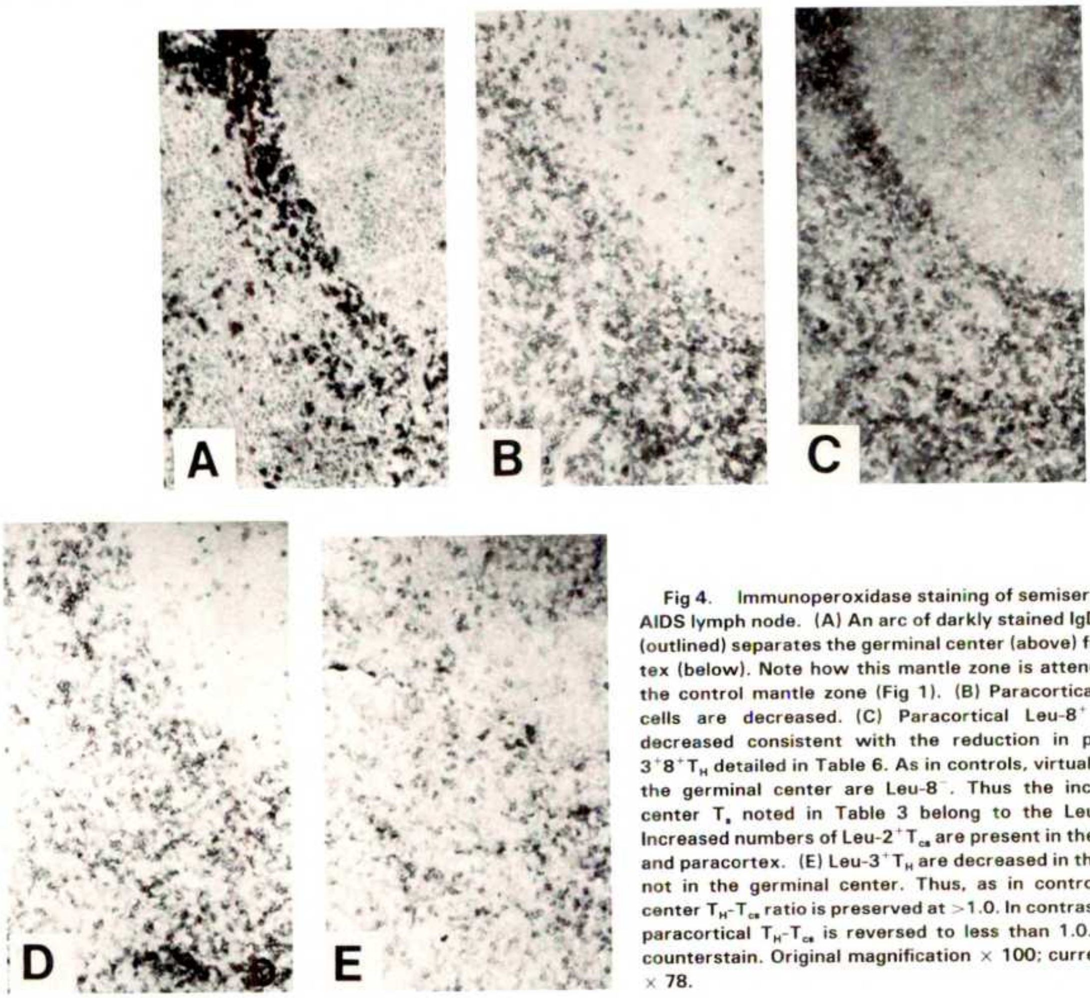

Fig 4. Immunoperoxidase staining of semiserial sections of an AIDS lymph node. (A) An arc of darkly stained IgD$^+$ mantle B cells (outlined) separates the germinal center (above) from the paracortex (below). Note how this mantle zone is attenuated relative to the control mantle zone (Fig 1). (B) Paracortical Leu-4^+ total T cells are decreased. (C) Paracortical Leu-8^+ cells are also decreased consistent with the reduction in paracortical Leu-3^+8^+ T_H detailed in Table 6. As in controls, virtually all cells within the germinal center are Leu-8^-. Thus the increased germinal center T_s noted in Table 3 belong to the Leu-8^- subset. (D) Increased numbers of Leu-2^+ T_{cs} are present in the germinal center and paracortex. (E) Leu-3^+ T_H are decreased in the paracortex but not in the germinal center. Thus, as in controls, the germinal center T_H-T_{cs} ratio is preserved at >1.0. In contrast to controls, the paracortical T_H-T_{cs} is reversed to less than 1.0. Methylene blue counterstain. Original magnification × 100; current magnification × 78.

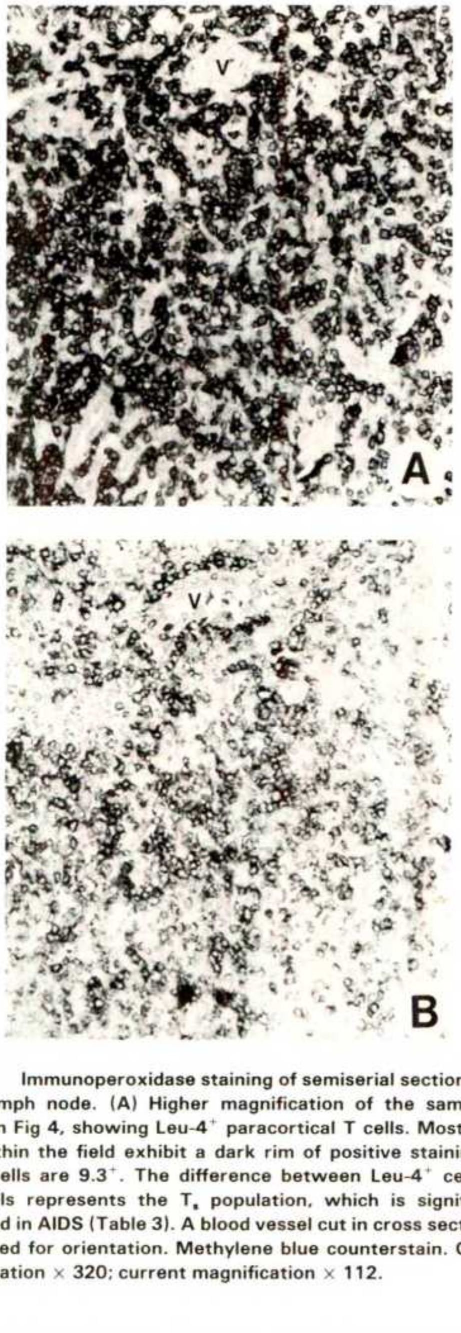

Fig 5. Immunoperoxidase staining of semiserial sections of an AIDS lymph node. (A) Higher magnification of the same case shown in Fig 4, showing Leu-4$^+$ paracortical T cells. Most of the cells within the field exhibit a dark rim of positive staining. (B) Fewer cells are 9.3$^+$. The difference between Leu-4$^+$ cells and 9.3$^+$ cells represents the T_s population, which is significantly increased in AIDS (Table 3). A blood vessel cut in cross section (V) is included for orientation. Methylene blue counterstain. Original magnification × 320; current magnification × 112.

As in the control group, T cells exhibited normal patterns of antigen expression in regard to the presence of Leu-1 and Leu-5 and the absence of Leu-6, OKT9, and OKT10. The majority of T cells were HLA-DR$^-$.

DISCUSSION

The most widely reported phenotypic abnormality in patients with AIDS, inversion of the Leu-3$^+$-Leu-2$^+$ ratio,[1-16] is a nonspecific finding that has been documented in a wide variety of clinical settings.[8-11,17-20,22,37-43] Interpretation of the Leu-3$^+$-Leu-2$^+$ ratio is complicated by the fact that anti–Leu-3(T4) antibodies react variably with monocytes, macrophages, and dendritic cells[31] and may thereby artifactually increase the ratio. In the present study, we omitted Leu-3$^+$ histiocytes from our counts of Leu-3$^+$ T cells and verified this by noting that the difference between Leu-4$^+$ and Leu-2$^+$ cell counts approximated the Leu-3$^+$ cell count (Table 5).

Another difficulty involving the Leu-3$^+$-Leu-2$^+$ ratio is that, although often referred to as the helper-suppressor ratio, it is actually the helper-cytotoxic/suppressor ratio, since anti–Leu-2(T8) stains T_c as well as T_s subsets.[28] The use of antibody 9.3 (which stains T_H and T_c but not T_s) in conjunction with other T cell markers allows dissection of T cells into separate T_H, T_c, and T_s phenotypes (Table 2). Using this principle, we documented marked differences between T_H-T_s and T_H-T_{cs} ratios (Table 3). These were more apparent in the paracortex of control lymph nodes than in AIDS lymph nodes, since in the latter most Leu-2$^+$ T cells were T_s, whereas in the former most Leu-2$^+$ cells were T_c.

Beyond assessment of the Leu-3$^+$-Leu-2$^+$ ratio, our quantitative immunohistologic analysis of lymph nodes from AIDS patients revealed that most of the alterations among T cell subsets were restricted to the paracortical T cell domain. In contrast, except for an increase in Leu-8$^-$ T_s, the germinal center B cell domain had a normal complement of T cells and a T_H-T_s ratio >1.0 (Table 7).

Because the quantitation of certain T cell subsets, including T_c, T_s, and those in Table 6, require indirect calculations, they are more prone to error than are direct measurements and should be regarded as approximations rather than as definitive values. Furthermore, knowledge of the full functional repertoire of the T cell subsets defined by 9.3 and anti–Leu-8 is still evolving. Nevertheless, it is possible to suggest certain correlations among T cell phenotypes and functions in AIDS in the context of our current quantitative data.

Our studies demonstrated that Leu-3$^+$8$^-$ T_H are intact within the germinal center B cell domain in AIDS. This subset is known to provide the major helper effect for B cell differentiation into immunoglobulin-secreting cells.[27] In addition, both Leu-2$^+$8$^+$ and Leu-2$^+$8$^-$ T cells are required to suppress this helper function.[27] While Leu-2$^+$ T_s are increased within the germinal center in AIDS, they are virtually all Leu-8$^-$. Thus they may not be optimal suppressors of certain B cell activities. In addition, the germinal center T_H-T_s ratio is relatively preserved at >1.0. Although we were unable to quantitate paracortical Leu-3$^+$8$^-$ T_H, the normal levels of this subset within germinal centers, together

Table 7. Microenvironmentally Specific Alterations of T Cell Subsets in AIDS

	Lymph Node Microenvironment	
Parameter	Germinal Center (B Cell)	Paracortex (T Cell)
No. of Leu-3$^+$8$^-$ (helper) T cells	Normal	Undetermined
No. of Leu-3$^+$8$^+$ (helper) T cells	Normal	Decreased
No. of 9.3$^+$, Leu-2$^+$ (cytotoxic) T cells	Normal	Decreased
No. of 9.3$^-$, Leu-2$^+$ (suppressor) T cells	Increased	Increased
T_H-T_{cs} and T_H-T_s ratios	>1.0	<1.0

From our data, it was not possible to determine whether Leu-3$^+$8$^-$ T cells were increased or decreased within the paracortex.

with the previously reported normal levels of peripheral blood Leu-$3^+8^-T_H$ in homosexual men with PGL,[18] suggest that normal levels of Leu-$3^+8^-T_H$ may also exist within the paracortex in AIDS. These microenvironmentally specific findings may help to explain the follicular hyperplasia and polyclonal hypergammaglobulinemia that is present in many homosexual patients.[1-8,10,11,18,29,36] One prior study of PGL patients noted a consistent reversal of the T_H-T_{cs} ratio to <1.0 within germinal centers.[23] This was not observed in any of our AIDS patients or in another study comparing the immunohistology of lymph nodes from AIDS and PGL patients.[9]

Nicholson et al[18] have demonstrated that the Leu-$3(T4)^+8^+$ subset is decreased in the peripheral blood of patients with PGL. Our study suggested a reduction of this same subset within the paracortex of lymph nodes from patients with AIDS (Table 6). Furthermore, we observed a reversal not only of the T_H-T_s ratio, but also of the T_c-T_s ratio (Table 3). Consistent with these findings, prior functional studies of peripheral blood cells have documented increased in vitro suppressor activity in patients with AIDS or PGL.[6,18] The Leu-$3^+8^+T_H$ subset is reactive in the autologous mixed lymphocyte reaction and has a major role in the induction of delayed type hyersensitivity and the induction of T_s.[18,27] Some of these T_s may act as intermediaries in the suppression of B cell immunoglobulin synthesis.[18,27] In light of these functional characteristics, the decreased Leu-$3^+8^+T_H$, decreased T_c, and increased T_s documented within the paracortex of the AIDS cases are consistent with the defective cell-mediated immunity documented previously in AIDS.[1-16] The hypergammaglobulinemia and plasmacytosis previously noted in such patients[1-11,18,29,36] may be related to defective induction of those T_s concerned with the down regulation of humoral immunity.

Within the paracortex, the AIDS patients exhibited reduced Leu-8^+ cells (Table 4). It has been proposed that quantitation of novel subsets in homosexual patients may be of greater value than Leu-3^+-Leu-2^+ ratios in predicting clinical outcome.[18] Results from the seven AIDS patients offer some support of this possibility when supplemented with unpublished data from two patients with PGL and one heterosexual man who died of posttransfusion AIDS. Despite similar Leu-3^+-Leu-2^+ ratios in these ten patients, the two terminal AIDS patients had the lowest levels of Leu-8^+ cells, whereas the two patients with PGL had the eighth and ninth highest levels of Leu-8^+ cells. Similar trends were noted for total (Leu-4^+) T cells.

Considerable evidence now exists that HTLV-III/LAV retrovirus, which is cytotropic and cytopathic for Leu-3^+T_H, is the etiologic agent of AIDS and PGL.[44,45] The Leu-3 antigen is an essential component of the cell surface receptor necessary for HTLV-III/LAV infection.[44] Recent evidence also indicates that the Leu-8 antigen may somehow be involved in retroviral reception and penetration.[44] This may help to explain our current data that suggest that Leu-$3^+8^+T_H$ are markedly depleted in AIDS.

It will be important to determine whether the T cell subset alterations detected with anti–Leu-8 and 9.3 in this study are unique to AIDS or are also typical of other diseases associated with a decreased Leu-3^+-Leu-2^+ ratio. These refinements in our ability to dissect and quantitate T cell subsets in situ may prove to be useful in establishing the diagnosis, prognosis, and pathogenesis of immunodeficiency diseases.

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Temperature-Dependent Effects of EDTA on the Membrane Glycoprotein IIb-IIIa Complex and Platelet Aggregability

by Dominique Pidard, Dominique Didry, Thomas J. Kunicki, and Alan T. Nurden

In agreement with previous studies, we observed that incubation of washed human platelets with EDTA at 37 °C for short periods caused an irreversible loss of their aggregation response to adenosine diphosphate and markedly diminished their capacity to bind fibrinogen. AP-2 is a monoclonal antibody that reacts with a determinant specific to the glycoprotein (GP) IIb-IIIa complex. We now report that in a direct binding assay, the number of sites for AP-2 on platelets incubated with EDTA at 37 °C fell to ~30% of those present on control platelets. This effect of EDTA was not observed at room temperature. Analysis of the treated platelets by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed normal amounts of GP IIb and GP IIIa. However, studies using crossed immunoelectrophoresis with ^{125}I-AP-2, ^{125}I-Tab (anti-GP IIb), or ^{125}I-AP-3 (anti-GP IIIa) in intermediate gels showed that at 37 °C, EDTA was inducing an irreversible change in GP IIb-IIIa complexes. A reduction in size and probable dissociation of the GP IIb-IIIa precipitate was accompanied by the appearance of precipitates having the characteristics of those given by free GP IIb and free GP IIIa and the location of a major new cathodal precipitate, which bound Tab and AP-3 but not AP-2. Membrane modifications associated with the loss of antigenic determinants on GP IIb-IIIa may explain EDTA-induced loss of platelet aggregability at 37 °C.

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PLATELETS ACTIVATED by adenosine diphosphate (ADP) and other physiologic stimuli express specific and saturable receptors for fibrinogen.[1] Binding of fibrinogen requires divalent cations and is correlated with the onset of platelet aggregation.[2-4] Growing evidence suggests that divalent cation-dependent complexes of two membrane glycoproteins (GP), IIb and IIIa, play an essential role in fibrinogen receptor expression and therefore in the aggregation process.[5-7] Part of this evidence has accumulated from studies with monoclonal antibodies such as AP-2[8] and T10,[9] which react with complex-dependent epitopes on GP IIb-IIIa and inhibit both fibrinogen binding to stimulated platelets and platelet aggregation. Other such antibodies have recently been catalogued.[10]

That GP IIb and GP IIIa may form complexes in the presence of Ca^{2+} was first shown in studies in which Triton X-100 extracts of platelets or isolated membranes were analyzed by crossed immunoelectrophoresis (CIE). A major immunoprecipitate was observed that, after divalent cation chelation with EDTA, was replaced by two new precipitates, one containing GP IIb and the other, GP IIIa.[5,11,12] Other studies on the extracted glycoproteins showed the complex to be a heterodimer[13,14] and that the complexes can bind ^{45}Ca^{2+},[15] possibly through GP IIb.[16] Indeed, they may represent the major calcium-binding site on the platelet surface[17,18] and participate in the transport of this cation across the plasma membrane.[19] Binding studies performed with the monoclonal antibodies AP-2[8] and T10[9] have confirmed the presence of the heterodimer in the membrane of inactivated platelets.

The results of studies by Zucker and Grant[20,21] showed that platelets incubated at 37 °C for short periods with EDTA subsequently failed to aggregate when challenged with ADP. The EDTA-treated platelets were unable to bind fibrinogen in the presence of ADP and exhibited a reduced capacity to bind ^{45}Ca^{2+}.[4,17] Recent advances in our knowledge of fibrinogen receptor expression suggest that EDTA may be acting on the GP IIb-IIIa heterodimers. To investigate this possibility, we have examined the effect of incubating platelets with EDTA at 37 °C, studying in parallel (a) platelet aggregation and fibrinogen binding, (b) AP-2 binding, and (c) GP IIb-IIIa stability. Results show that at 37 °C and alkaline pH, EDTA directly influences the structure of the IIb-IIIa heterodimers in the platelet membrane.

MATERIALS AND METHODS

The following materials were obtained commercially: prostaglandin E$_1$ (PGE$_1$), grade I apyrase, bovine serum albumin (BSA, fraction V), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), ADP, phenylmethylsulfonyl fluoride (PMSF), and synthetic hemisulfate leupeptin were from Sigma Chemical Co (St Louis); ethylenediamine tetraacetic acid disodium salt (EDTA) from Prolabo (Paris); purified human fibrinogen from Kabi Diagnostics (Stockholm); ^{125}I-fibrinogen was obtained from the Service des Radioisotopes of the Centre National de Transfusion Sanguine (Paris); Na^{125}I from the Centre de l'Energie Atomique (Orsay, France); agarose HSA type from Litex (Glostrup, Denmark); Triton X-100 from BDH Chemicals Ltd (Poole, England); glycine, tris (hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), acrylamide, bisacrylamide, ammonium persulfate, 2-mercaptoethanol, TEMED, and Coomassie blue R-250 (CB-R) from Bio-Rad (Richmond, Calif). All other chemicals were of reagent grade. The monoclonal antibodies Tab[9,22] and AP-3[23] were kindly provided as purified IgG by Dr R. McEver (The University of Texas Health Science Center, San Antonio, Tex) and Dr P.J. Newman (The Blood Center of Southeastern Wisconsin, Milwaukee), respectively.

Platelet isolation. Blood (6 vol) was obtained from adult human volunteers and anticoagulated with acid-citrate-dextrose anticoagulant (ACD; NIH formula A) (1 vol).[24] Platelet-rich plasma prepared by centrifugation at 20 °C for 15 minutes at 120 g was acidified to pH 6.5 by addition of a one tenth vol ACD-A. PGE$_1$ (100 nmol/L) and apyrase (25 µg/mL) were also added. Washed platelets were prepared by the method of Patscheke[24] as modified by Legrand et al.[25] In the standard procedure, the platelets were washed in 36 mmol/L citric acid, 5 mmol/L glucose, 5 mmol/L KCl, 2 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 103 mmol/L NaCl, 3.5 mg/mL BSA, 100 nmol/L PGE$_1$, 25 µg/mL apyrase, pH 6.5 (washing buffer). Unless otherwise stated, the platelets were finally resuspended in a modified

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Tyrode's solution consisting of 137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L $NaHCO_3$, 0.3 mmol/L NaH_2PO_4, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5.5 mmol/L glucose, 5 mmol/L HEPES, pH 7.4 (HBMT).

Incubation with EDTA. Twice-washed platelets were resuspended at 10^9/mL in HBMT or the same buffer lacking calcium and magnesium but containing 2 mmol/L EDTA (HBMT-EDTA). In some experiments, the concentration of EDTA was varied as detailed in the text. Both buffers also contained 100 nmol/L PGE_1 and 25 μg/mL apyrase. The platelet suspensions were incubated at 20 °C or 37 °C for periods of up to 15 minutes in a thermostatically controlled water bath. Control incubations were performed in HBMT alone. After the incubation, the platelet suspensions were diluted with 5 vol washing buffer, centrifuged, and the platelets were resuspended at room temperature in HBMT containing 3.5 mg/mL BSA but lacking apyrase and PGE_1. To standardize the experimental conditions, all subsequent studies were started 30 minutes after the final platelet resuspension. Such platelets are subsequently referred to as "pretreated" platelets.

Platelet aggregation. Normal or pretreated washed platelets in HBMT were adjusted to 3×10^8 platelets per milliliter. Platelet aggregation was studied photometrically using a Labintec aggregometer (Central Labo, Paris). The platelet suspension (0.36 mL) was stirred (1,100 rpm) at 37 °C in the presence of 0.2 mg/mL fibrinogen and 5 or 10 μmol/L ADP. Changes in light transmission were continuously recorded and compared with the maximal change observed with HBMT buffer alone.

Fibrinogen binding assay. The binding of purified human ^{125}I-fibrinogen to ADP-stimulated control and EDTA-pretreated platelets was measured according to the procedure of Legrand et al.[26] The characteristics of the ^{125}I-fibrinogen preparation have already been reported.[26] Briefly, the assay was performed as follows: 0.4-mL vol of a washed platelet suspension in HBMT (5 to 8×10^8 platelets per milliliter) were mixed with 80 μL of HBMT and 40 μL of a 12 μmol/L solution of ^{125}I-fibrinogen in HBMT to give a final concentration of 1 μmol/L, at which steady-state binding of fibrinogen to its platelet receptor reaches saturation under our experimental conditions.[26] Binding was initiated three minutes later by adding 30 μL of a 200-μmol/L solution of ADP dissolved in HBMT to give a final concentration of 10 μmol/L, and continued for 30 minutes at room temperature, before triplicate samples of 0.15 mL were layered onto 0.5 mL of an inert oil mixture ($d = 1.014$).[26] The samples were centrifuged for three minutes at 12,000 g in an Eppendorf model 5414 centrifuge (Brinkman Instruments, Inc, Westbury, NY). Supernatants and pellets were fractionated and their associated ^{125}I-radioactivity measured in a Gamma 7000 counter (Beckman Instruments, Inc, Fullerton, Calif). Nonspecific binding of ^{125}I-fibrinogen was defined as that observed in the presence of a 20-fold excess of unlabeled fibrinogen initially added in a 80-μL vol instead of the buffer alone (see above). It represented approximately 35% of the total binding, from which it was substracted to give the specific binding.

AP-2 binding assay. AP-2 was isolated from ascites fluid and the monoclonal IgG labeled with ^{125}I using the chloramine T method.[8] The assay for the direct binding of ^{125}I-labeled antibody to washed platelets was adapted from that previously used by us.[8] Briefly, normal or pretreated washed platelet suspensions were first adjusted to 10^8 platelets per milliliter with HBMT. In the standard assay, aliquots (0.3 mL) were then mixed with near-saturating concentrations of ^{125}I–AP-2 (≥3 μg/mL). For Scatchard analysis (see ref. 8), aliquots were mixed with increasing amounts of ^{125}I–AP-2 (0.5 to 5.0 μg/mL) as detailed in the text. After a 30-minute incubation at room temperature, 0.14-mL samples were layered in duplicate over 0.5 mL 20% (wt/vol) sucrose in HBMT. Platelets were pelleted by centrifugation at 12,000 g for two minutes in an Eppendorf centrifuge. Each supernatant was aspirated and the radioactivity in both supernatants and pellets measured in a Beckmann 7000 gamma counter. Nonspecific binding was measured in the presence of a 50-fold excess of unlabeled AP-2. As this was found to be negligible (≤3% of total binding), the data is presented as total binding.

Crossed immunoelectrophoresis. Pretreated platelets resuspended in HBMT were diluted with 5 vol washing buffer and the platelets sedimented. The pellets were resuspended in 0.1 mol/L glycine, 0.038 mol/L Tris, pH 8.7 (Tris-glycine) prechilled to 4 °C. Platelet solubilization was performed at 4 °C by incubation for 30 minutes with 1% (vol/vol) Triton X-100. In selected experiments, the solubilization step was performed in the additional presence of 500 μg/mL leupeptin and 1 mmol/L PMSF. The Triton X-100 insoluble material was removed by ultracentrifugation and the supernatants stored at −80 °C until use. CIE was performed according to the procedures detailed by Kunicki et al[5] and Pidard et al.[8] Briefly, first-dimension electrophoresis was performed at 10 V/cm for one hour in 1% (wt/vol) agarose containing 0.5% (vol/vol) Triton X-100 in Tris-glycine. Second-dimension electrophoresis was performed at 2 V/cm for 18 hours into a biphasic gel system consisting of (a) an intermediate gel containing 1% agarose, 0.5% Triton X-100 and, on occasion, trace amounts of ^{125}I–AP-2, ^{125}I-Tab or ^{125}I–AP-3 and (b) an upper gel containing precipitating concentrations of a polyspecific rabbit antihuman platelet antibody preparation. Further experimental details are given in the figure legends. Immunoprecipitates were located by Coomassie blue R250 (CB-R) staining, and those containing ^{125}I, by autoradiography.[5,8]

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Pretreated platelets in HBMT were pelleted and resuspended at 2×10^9/mL in 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L EDTA, pH 7.0, and solubilized by heating at 100 °C for five minutes after the addition of a one fifth vol of 12% (wt/vol) SDS containing 30 mmol/L *N*-ethylmaleimide. Aliquots of Triton X-100–soluble platelet extracts were diluted with an equal vol of 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L EDTA, pH 7.0, before being treated similarly. Triton X-100–insoluble material was resuspended in the same buffer before SDS solubilization. Samples were prepared with (reduced) or without (nonreduced) disulfide reduction, and electrophoresis was performed using 7% or 6% acrylamide rod gels, or 7% to 12% gradient acrylamide slab gels, according to the procedures detailed by Nurden et al.[27] Glycoproteins were located by periodic-acid Schiff (PAS) staining, and proteins, by CB-R staining. Densitometric scanning of PAS-stained rod gels was performed using a Vernon Integrating Photometer (Vernon Integrating Photometers Ltd, Paris). In some experiments, membrane glycoproteins were labeled with ^{125}I using the lactoperoxidase-catalyzed procedure detailed by Nurden et al[27] before the platelets were exposed to EDTA. ^{125}I-labeled polypeptides were revealed by autoradiography of dried polyacrylamide slab gels as previously described by us.[27]

RESULTS

Experimental conditions. The aim of this study was to examine the effect of controlled incubation with EDTA on the platelet aggregation response and on the GP IIb–IIIa complex. All platelet washing steps were therefore performed in the presence of divalent cations but at pH 6.5, to prevent the formation of platelet aggregates during centrifugation. Incubations at 37 °C were performed at pH 7.4 in the presence of (a) apyrase to prevent platelets becoming refractory to released ADP[28] and (b) PGE_1 to help maintain platelet integrity and inhibit granule release.[29]

Platelet aggregation. Washed human platelets that had been incubated for 15 minutes at 37 °C with 2 mmol/L EDTA failed to aggregate when resuspended in HBMT containing divalent cations and stimulated by ADP in the presence of added fibrinogen. A typical experiment is shown in Fig 1. Platelets that were incubated at 37 °C under control conditions in the presence of 2 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ were normally aggregated. Incubation of the platelets at 37 °C with equimolar amounts of Ca^{2+} and EDTA (CaEDTA-treated platelets) did not affect platelet aggregability. This suggested that the primary effect of EDTA was to chelate membrane-bound calcium. The phenomenon was time and temperature dependent. Incubation of platelets with 2 mmol/L EDTA for 15 minutes at 20 °C did not result in a loss of aggregability. Incubation of platelets with 2 mmol/L EDTA for shorter periods at 37 °C resulted in partial inhibition of the aggregation response (not shown). We also consistently observed a reduced "swirling" phenomenon in the final suspensions of unstimulated EDTA-treated platelets when compared with the control preparations. This may indicate a loss of their discoid shape during exposure to EDTA at 37 °C, a phenomenon previously described for EDTA-treated platelets by White.[30] In agreement with this conclusion, aggregation studies performed at lower concentrations of EDTA-treated platelets (1×10^8/mL) did not show the typical decrease in light transmission that normally follows the addition of ADP to stirred platelet suspensions and that reflects the change from disk-shaped to spherical platelets with pseudopods (not shown).

Fibrinogen binding. Control and EDTA-treated platelets were next compared as to their ability to bind ^{125}I-fibrinogen when activated with 10 μmol/L ADP in HBMT containing divalent cations. In experiments performed using platelets from four donors, the specific ADP-induced binding of fibrinogen to platelets preincubated at 37 °C under control conditions was 372 ± 130 ng fibrinogen per 10^8 platelets. In contrast, platelets of the same donors preincubated with 2 mmol/L EDTA for 15 minutes at 37 °C bound only 162 ± 90 ng fibrinogen per 10^8 platelets (mean ± SD). Thus fibrinogen binding was reduced to approximately 43% of its normal value.

Monoclonal antibody binding. Platelets prepared for aggregation studies were also tested for their capacity to bind the monoclonal antibody AP-2. Using near-saturating concentrations of ^{125}I–AP-2,[8] we found that platelets preincubated at 37 °C for 15 minutes with 2 mmol/L EDTA bound only 29.7% of the amount of antibody that bound to platelets incubated at 37 °C under control conditions (Table 1). No such reduction was observed when platelets were treated with CaEDTA at 37 °C or with EDTA at 20 °C. Increasing the concentration of EDTA (10 mmol/L) or the duration of the incubation at 37 °C caused only a small additional fall in the number of AP-2 binding sites. For example, approximately 20% of the binding sites remained on platelets pretreated with 2 mmol/L EDTA for 45 minutes at 37 °C (data not shown).

Scatchard analysis after the incubation of washed platelets with increasing concentrations of ^{125}I–AP-2 (see Materials and Methods) showed that the platelets bound 39,388 ± 5,832 IgG molecules per platelet with an affinity Kd = 0.81 ± 0.21 nmol/L (mean ± SD, n = 9). These values were similar to those previously reported by us.[8] Preincubation of the washed platelets at 37 °C for 15 minutes under the control conditions did not significantly change the platelet capacity to bind AP-2 (Table 1). In a single experiment, platelets that had been treated with 2 mmol/L EDTA for 15 minutes at 37 °C were also incubated with varying concentrations of ^{125}I–AP-2. Here, Scatchard analysis showed that the number of AP-2 molecules that had bound had fallen to 17,950 per platelet with an affinity Kd = 1.02 nmol/L. The latter value was unchanged from that obtained for untreated platelets, thus EDTA was primarily affecting the number of AP-2 receptors.

Crossed immunoelectrophoresis. Platelets prepared for aggregation studies were also solubilized with 1% Triton X-100 and the soluble extracts analyzed by CIE. Initial experiments established that the pattern of immunoprecipitates obtained for platelets incubated at 37 °C under the control conditions was identical to that obtained for washed

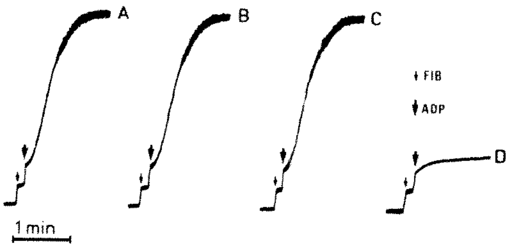

Fig 1. Irreversible inhibition of platelet aggregation by EDTA at 37 °C. Washed human platelets were incubated for 15 minutes (A) + 2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+} at 37 °C; (B) + 2 mmol/L Ca^{2+}, 2 mmol/L EDTA at 37 °C; (C) + 2 mmol/L EDTA at 20 °C; and (D) + 2 mmol/L EDTA at 37 °C. The platelets were sedimented and resuspended at 3×10^8 platelets per milliliter in HBMT. After 30 minutes, aliquots were tested for platelet aggregation induced by 10 μmol/L ADP in the presence of 0.2 mg/mL fibrinogen. Typical aggregation curves are shown.

Table 1. ^{125}I–AP-2 Binding to Pretreated Washed Platelets

Conditions of Incubation (15 min)	Amount of bound ^{125}I–AP-2 ng IgG/10^8 Platelets	 Percentage
2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+}; 20 °C	1,044 ± 126 (n = 3)	100.0
2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+}; 37 °C	1,176 (n = 1)	112.6
2 mmol/L Ca^{2+}, 2 mmol/L EDTA; 37 °C	1,130 (n = 2)	108.2
2 mmol/L EDTA; 20 °C	1,082 (n = 1)	103.6
0.5 mmol/L EDTA; 37 °C	330 (n = 2)	31.6
2.0 mmol/L EDTA; 37 °C	310 ± 97 (n = 4)	29.7
10.0 mmol/L EDTA; 37 °C	286 (n = 1)	27.4

The treated platelets were sedimented and resuspended at 1×10^8 platelets per milliliter in HBMT for the ^{125}I–AP-2 binding assay performed using ≥3 μg/mL AP-2 (see Materials and Methods). Results are expressed as the mean ± SD (where calculated) and as the percentage of the control incubation at 20 °C arbitrarily designated as 100%.

platelets prepared without pretreatment. In contrast, as shown in Fig 2, analysis of platelets that had been incubated for 15 minutes with 2 mmol/L EDTA at 37 °C revealed a marked reduction in the size of the immunoprecipitate given by the GP IIb–IIIa complex and the presence of a new, weakly stained precipitate in a more cathodal position (see next section). The use of small amounts of ^{125}I–AP-2 in the intermediate gel confirmed that the former precipitate contained GP IIb–IIIa but that no new AP-2 binding immunoprecipitates were present after the EDTA treatment. The area occupied by the GP IIb–IIIa precipitate on each plate was measured by planimetry and expressed relative to that of another major membrane antigen previously identified as GP IIIb.[5] The size and form of the GP IIIb containing immunoprecipitate was unchanged after platelet incubation with EDTA, and it therefore served as an internal standard. Table 2 shows that for three experiments, the apparent GP IIb–IIIa content of EDTA-treated platelets was about 39% of that present in platelets incubated under the control conditions. No such reduction was seen for platelets treated with EDTA at 20 °C or CaEDTA at 37 °C. A number of other immunoprecipitates located by CB-R staining are also identified in Fig 2. Neither GP Ib, highly sensitive to degradation by proteolytic enzymes,[31] nor α-granule proteins, such as fibrinogen, von Willebrand factor, and thrombospondin, appeared to have been modified during the platelet incubation at 37 °C. However, as an extra precaution against proteolysis, both the 37 °C pretreatment and the Triton X-100 solubilization steps were repeated in the additional presence of 500 μg/mL leupeptin and 1 mmol/L PMSF. The same apparent reduction in the GP IIb–IIIa content of the EDTA-treated platelets was observed.

Modification of GP IIb–IIIa in intact platelets by EDTA. Platelets that had been incubated with EDTA at 37 °C were solubilized with SDS and their glycoprotein composition analyzed by SDS-PAGE, followed by PAS staining. Densitometric scanning revealed that the GP IIb and GP IIIa content of EDTA-treated platelets was identical to that of platelets incubated at 37 °C under control conditions (data not shown). Thus EDTA was not causing the release of GP IIb or GP IIIa from the platelet surface. SDS-PAGE of the Triton X-100–soluble and –insoluble fractions also confirmed that GP IIb and GP IIIa were being normally solubilized by Triton X-100 from the EDTA-treated platelets.

One possible explanation for the effect of EDTA on AP-2 binding (Table 1) and platelet GP IIb–IIIa content (Fig 2) is that divalent cation chelation at 37 °C induces a dissociation of the complex in the platelet membrane. To investigate this possibility, CIE studies were performed using ^{125}I-Tab or ^{125}I–AP-3 in the intermediate gel. Tab is able to bind to free GP IIb,[9,22] and AP-3 reacts with free GP IIIa.[23] These antibodies, therefore, differ from AP-2, which recognizes a complex-dependent determinant.[8] Initial studies using ^{125}I-

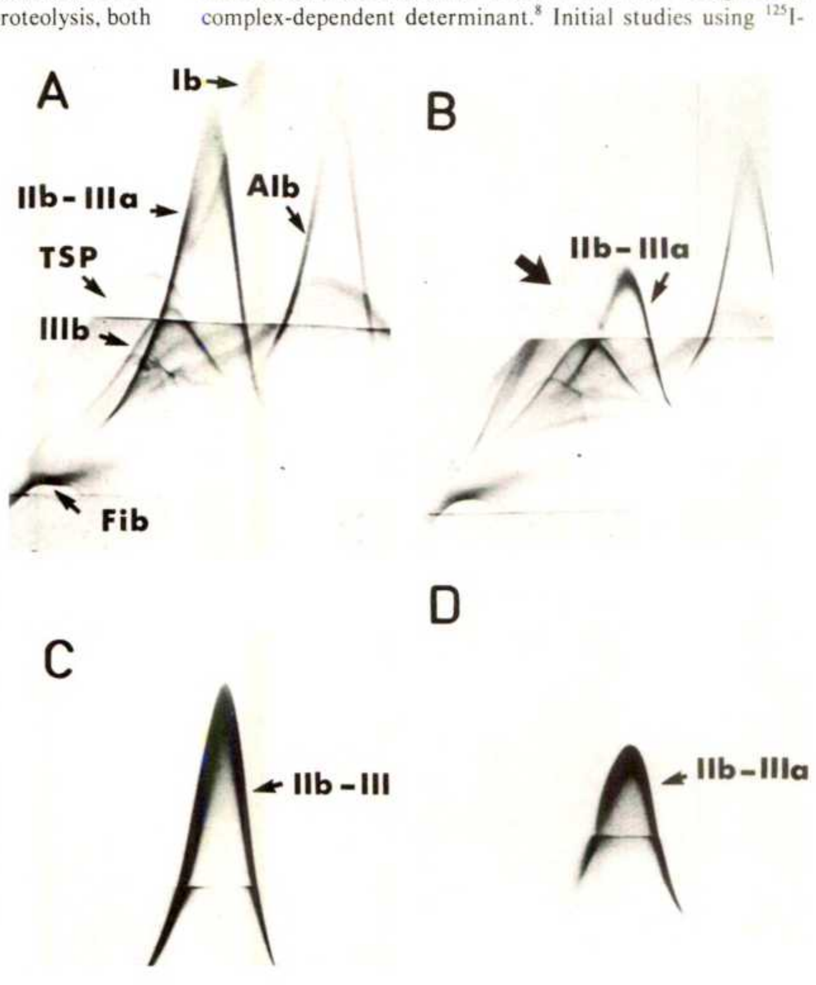

Fig 2. Reduced size of the GP IIb–IIIa immunoprecipitate on analysis of platelets incubated with EDTA at 37 °C. Washed human platelets were incubated for 15 minutes (A, C) + 2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+} at 37 °C, and (B, D) + 2 mmol/L EDTA at 37 °C. After sedimentation and resuspension in HBMT for 30 minutes, aliquots were prepared for CIE analysis (see Materials and Methods). Seventy micrograms of platelet protein solubilized in 1% Triton X-100 was electrophoresed from left to right in the first dimension. Electrophoresis in the second dimension was through an intermediate gel containing 3.5 × 10^5 cpm ^{125}I–AP-2. The upper gel contained 0.75 mg/cm^2 of polyclonal rabbit antihuman platelet antibody. In (A) and (B), immunoprecipitates were located by CB-R staining, and in (C) and (D), GP IIb–IIIa complexes that had bound AP-2 were detected by autoradiography. Note on (B) the appearance of a new precipitate, indicated by the heavy arrow. Alb, albumin; Fib, fibrinogen; TSP, thrombospondin.

Table 2. Quantitative Analysis of the GP IIb–IIIa Precipitate Obtained After CIE of Pretreated Washed Platelets

Conditions of Incubation (15 min)	Comparative Size of GP IIb–IIIa Precipitate	
	Ratio of GP IIb–IIIa to GP IIIb	Percentage
2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+}; 20 °C	2.92 ± 0.17 (n = 3)	100.0
2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+}; 37 °C	3.14 (n = 1)	107.5
2 mmol/L Ca^{2+}, 2 mmol/L EDTA; 37 °C	2.77 (n = 2)	94.9
2 mmol/L EDTA; 20 °C	3.68 (n = 1)	126.0
0.5 mmol/L EDTA; 37 °C	1.05 (n = 1)	36.0
2.0 mmol/L EDTA; 37 °C	1.13 ± 0.01 (n = 4)	38.7
10.0 mmol/L EDTA; 37 °C	0.88 (n = 1)	30.1

CIE of Triton X-100 extracts was performed as described in Fig 2. The area of the GP IIb–IIIa and GP IIIb precipitates was measured by planimetry and expressed as a ratio. Results are the mean ± SD (where calculated) and as the percentage of the control incubation at 20 °C arbitrarily designated as 100%.

Tab confirmed that only GP IIb–IIIa complexes were present in Triton X-100 extracts of platelets preincubated at 37 °C under control conditions (Fig 3) or with EDTA at room temperature (not shown). In contrast, when extracts of platelets that had been treated with EDTA at 37 °C were analyzed, two, or sometimes three, immunoprecipitates were labeled with the antibody. The first of these corresponded to that given by residual GP IIb–IIIa complexes and was identical to that which bound AP-2 (see Fig 2). The second was the new cathodal immunoprecipitate previously noted to be present on CB-R–stained gels and that clearly shows a line of antigenic identity to GP IIb–IIIa (Fig 3B) but which did not bind AP-2. This was a surprise, for previous studies had shown dissociated GP IIb to be present exclusively in an immunoprecipitate located directly above that containing residual GP IIb–IIIa.[5] Such an immunoprecipitate was occasionally observed (open arrow in Fig 3B). However, its presence was often difficult to confirm due to the intense labeling of the residual GP IIb–IIIa precipitate by ^{125}I-Tab. Figure 3 also shows CIE studies performed with ^{125}I-Tab in the intermediate gel when Triton X-100 extracts of the EDTA-treated platelets were made 5 mmol/L with EDTA and further incubated for 15 minutes at room temperature just before the CIE. These are conditions known to induce a dissociation of Triton X-100–solubilized GP IIb–IIIa heterodimers.[5] The precipitate containing free GP IIb[5] was now increased in size (Fig 3C), while that given by the residual GP IIb–IIIa complexes was decreased. By continuing the second incubation at room temperature for one hour, a total dissociation of residual GP IIb–IIIa was obtained (not shown). However, no modification in either the location, the shape, or the ^{125}I-Tab labeling intensity of the new cathodal precipitate was observed under these conditions.

^{125}I–AP-3 also labeled three immunoprecipitates during CIE of extracts of platelets incubated with EDTA at 37 °C (Fig 3D). The major arc corresponded to that given by residual GP IIb–IIIa complexes. Of the two cathodal arcs reacting with ^{125}I–AP-3, one was identified as the new GP IIb–IIIa-related precipitate also labeled by ^{125}I-Tab. This

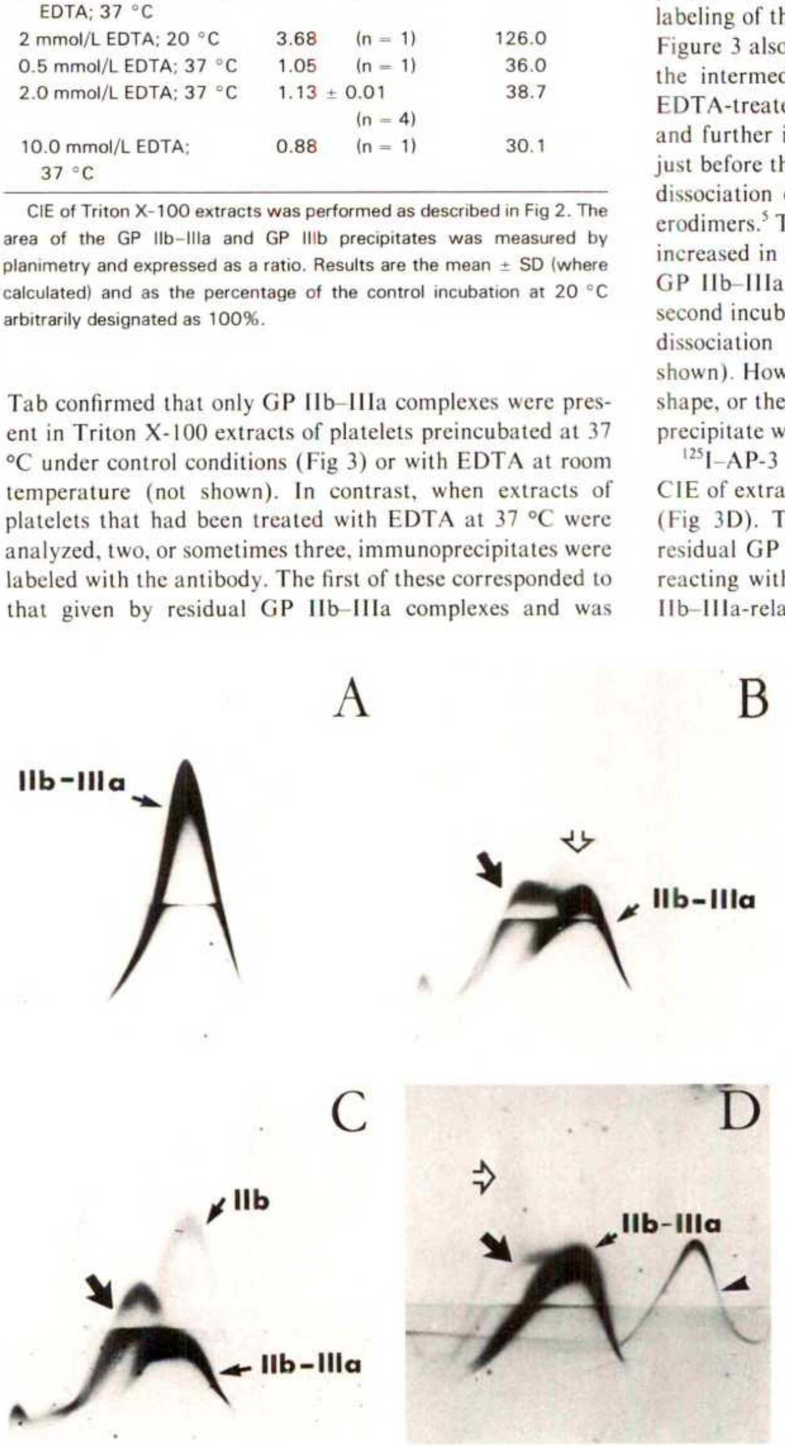

Fig 3. Temperature-dependent alterations in GP IIb–IIIa complexes by EDTA. Washed human platelets were incubated for 15 minutes at 37 °C (A) + 2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+} or (B, C, D) + 2 mmol/L EDTA and processed as described in Fig 2. CIE was performed with 3.5 × 10^5 cpm ^{125}I-Tab (A, B, C) or ^{125}I–AP-3 (D) in the intermediate gel. In (C), an identical Triton X-100–soluble extract to that in (B) was further incubated with 5 mmol/L EDTA at 20 °C for 15 minutes immediately before the first-dimension electrophoresis. Typical autoradiographs are shown. In (B) and (D), the open arrows indicate the location of free GP IIb and free GP IIIa, respectively. The arrow head in (D) points to the precipitate given by platelet albumin that was visualized as a result of traces of ^{125}I-labeled serum albumin that contaminated the ^{125}I–AP-3 preparation.

conclusion was based on its size, shape, and line of partial antigenic identity with the IIb-IIIa precipitate (compare Fig 3B and D). The third component (open arrow in Fig 3D) exhibited the features previously reported for free GP IIIa on CIE analysis, ie, a slower electrophoretic mobility than the GP IIb–IIIa heterodimer, leading to a more cathodal precipitate[5,23] and an absence of reactivity with ^{125}I-Tab[9] and ^{125}I–AP-2.[8]

The presence of both GP IIb and GP IIIa within the new cathodal immunoprecipitate was confirmed by SDS-PAGE analysis after excision and SDS solubilization of this particular precipitation line from unstained agarose gels, according to the procedure previously detailed by Kunicki et al.[5] The study was performed with Triton X-100 extracts of platelets whose surface proteins had been labeled with ^{125}I by the lactoperoxidase-catalyzed procedure before their incubation with EDTA at 37 °C. Autoradiograms of dried polyacrylamide gels revealed the presence of two ^{125}I-labeled components in this new immunoprecipitate, with nonreduced and reduced molecular weights identical to those of GP IIb and GP IIIa (not shown).

DISCUSSION

It is well known that divalent cations are essential cofactors of platelet aggregation. Zucker and Grant[20,21] highlighted a special case when they described how pretreatment of platelet-rich plasma with EDTA at 37 °C resulted in an irreversible loss in the platelet aggregation response to ADP. This effect of EDTA was temperature dependent, occurring at 37 °C but not at 20 °C, and was only observed at alkaline pH (>pH 7.2). Aggregation induced by epinephrine and the ionophore A 23187 was also inhibited, and although the treated platelets were aggregated by thrombin, the velocity of aggregation was decreased. The subsequent observations that washed platelets incubated with EDTA at 37 °C (a) had a reduced capacity to bind $^{45}Ca^{2+}$[17] and (b) bound little or no fibrinogen in the presence of ADP and calcium[4] suggested that EDTA was influencing a crucial step at a late stage of the aggregation mechanism.

In confirming the temperature-dependent inhibitory effect of EDTA on the platelet aggregation response, we have shown that it is accompanied by structural changes in the GP IIb–IIIa complex. One manifestation of these changes was an irreversible fall in the number of AP-2 binding sites expressed by platelets treated with EDTA at 37 °C and alkaline pH. Significantly, this fall was of similar magnitude to the loss in the ability of the platelets to bind fibrinogen after ADP stimulation. The binding of AP-2 to GP IIb-IIIa complexes of normal platelets is followed by the inhibition of both platelet aggregation and fibrinogen binding after platelet activation.[8] As increasing evidence suggests that GP IIb–IIIa complexes constitute the fibrinogen receptor on the platelet surface,[1,6-9] it is probable that the reduced binding of both AP-2 and fibrinogen after incubation of platelets with EDTA at 37 °C are related events.

Our results with AP-2 may be compared with those reported by Coller et al,[32] using the monoclonal antibody 10E5, and those of Brass et al,[33] who used another antibody, A2A9. Both 10E5 and A2A9 appear to react with complex-dependent determinants on GP IIb–IIIa. Coller et al[32] observed a 51% reduction in the amount of 10E5 that bound to platelets after citrated platelet-rich plasma had been incubated with EDTA at pH 8.3 for 15 minutes at 37 °C. In contrast, Brass et al[33] reported a complete loss in the binding of A2A9 to gel-filtered platelets incubated with EDTA for one hour at 37 °C and pH 7.4. However, the binding of AP-2, 10E5, or A2A9 was not affected by EDTA when the temperature and pH of the incubation medium were kept within the ranges of 20 to 25 °C and pH 7.4 to 7.8, respectively.[8,32,33] Nonetheless, under these conditions, the binding of a newly described monoclonal antibody, 7E3, was decreased by more than 40%.[32] Finally, the binding of another anti-GP IIb–IIIa monoclonal antibody, T10, was reduced after the incubation of gel-filtered platelets for 30 minutes at room temperature and at pH 8.5.[9] Subtle differences in the specificity of the different antibodies for their determinants on GP IIb–IIIa probably account for the variations, which may also reflect progressive changes in the conformation or organization of the complexes during platelet incubation with EDTA at different pH and temperatures.

CIE experiments confirmed a direct temperature-dependent effect of EDTA on GP IIb–IIIa complexes in the platelet membrane. This was first observed as a reduction in the size of the immunoprecipitate containing GP IIb–IIIa. The good correlation between the extent of the EDTA-induced loss of AP-2 binding sites on intact platelets at 37 °C and the reduction in size of the GP IIb–IIIa precipitate strongly suggests that the changes observed after CIE did not arise subsequent to the platelet solubilization step, a view also supported by the fact that solubilization with Triton X-100 was performed under conditions that might stabilize preexisting IIb–IIIa heterodimers, ie, at low temperature and in the presence of Ca^{2+} released from disrupted organelles.[5]

The labeling by both Tab and AP-3 of a second major immunoprecipitate in a more cathodal position to GP IIb–IIIa during CIE of Triton X-100–soluble extracts of platelets that had been preincubated with EDTA at 37 °C was unexpected. These monoclonal antibodies react with EDTA-resistant determinants on GP IIb[9,22] and GP IIIa,[23] respectively. The results of studies performed after the incubation of Triton X-100–soluble platelet extracts with EDTA at 4 °C or 20 °C have shown that free GP IIb migrated at a rate similar to that of the GP IIb–IIIa complexes in the first-dimension electrophoresis, while dissociated GP IIIa gave a more cathodal immunoprecipitate.[5,11,12] Furthermore, any decrease in the precipitate given by GP IIb–IIIa due to a dissociation of the complex was directly compensated for by an increase in the arcs given by free GP IIb and free GP IIIa.[5] However, although small amounts of free GP IIb and free GP IIIa were visualized on CIE analysis of platelets pretreated with EDTA at 37 °C, the bulk of GP IIb and GP IIIa not present in the heterodimer precipitate was located within the new cathodal arc. Re-addition of EDTA to the Triton X-100–soluble extracts prepared from platelets pretreated with EDTA at 37 °C resulted in dissociation of residual GP IIb–IIIa complexes and the increased appearance of ^{125}I-Tab- or ^{125}I-AP-3-labeled arcs in the position of free GP IIb and free GP IIIa (data not presented). In contrast, the antigens within the new major cathodal immunoprecipitate were not sensitive to further EDTA treatment. Thus it appears that this precipitate contains GP IIb and GP IIIa reorganized into a new common structure that specifically forms under conditions of low calcium concentration and elevated temperature and that lacks the IIb–IIIa heterodimer-specific epitopes recognized by antibodies such as AP-2. The fact that the glycoproteins within this precipitate have a more cathodal migration is suggestive of polymer formation. That this may be so is also suggested by the parallel studies of Shattil et al[34] and Fitzgerald and Phillips.[35] These authors have described populations of GP IIb–IIIa complexes solubilized from platelets treated with EDTA at 37 °C that showed electrophoretic properties in polyacrylamide gels under nondenaturing conditions[34] or sedimentation profiles after centrifugation across sucrose density gradients[35] that were indicative of the presence of high-molecular-weight aggregates.

The temperature dependence of the EDTA effect is striking. As temperature has only a limited effect on the chelating capacity of EDTA (see ref. 19), it appears that another explanation is required. One possibility is that some of the Ca^{2+} binding sites located on GP IIb or GP IIIa[15-18] are only accessible to EDTA at 37 °C, perhaps due to temperature-dependent membrane fluidity changes.[36] This may then lead to dissociation of GP IIb–IIIa complexes, followed by the recombination of the monomeric glycoproteins into high-molecular-weight aggregates lacking the structural specificities of the heterodimers. Alternative effects of EDTA also cannot be excluded. These could involve the undetected solubilization of loosely bound peripheral membrane proteins important for GP IIb–IIIa organization or stimulation of membrane enzymatic activities leading to degradation or cross-linking of GP IIb and GP IIIa. However, the observed lack of inhibition by leupeptin and the normal migration of GP IIb and GP IIIa on SDS-PAGE would seem to rule out both an effect of the major platelet Ca^{2+}-dependant protease[31,37] and a covalent bonding of separated polypeptide chains.

In conclusion, our studies point to a temperature-dependent effect of EDTA on GP IIb–IIIa complexes, which may explain the loss of aggregability of the treated platelets through their inability to bind fibrinogen after platelet activation. As such, our results differ from those recently presented by Zucker et al,[38] who also examined the behavior of GP IIb and GP IIIa in Triton X-100–soluble extracts of human platelets incubated with EDTA at 37 °C. These authors showed the presence of GP IIb–IIIa complexes in Triton X-100 extracts of membranes prepared from the EDTA-treated platelets but did not quantitate the levels of GP IIb–IIIa present or examine the intact platelets in antibody binding studies. The effect of EDTA on GP IIb–IIIa appears to be subtle. However, the changes appear to concern functionally important regions of the complex, and thus EDTA treatment may represent a useful approach to elucidating the role of this complex in the responses of platelets to activation.

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Pathogenesis of B Cell Lymphoma in a Patient With AIDS

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Lymphoma occurs at increased frequency in patients with the acquired immunodeficiency syndrome (AIDS). We studied, using serologic and molecular techniques, one such lymphoma for (a) evidence of infection with human T lymphotropic virus, type III (HTLV-III), and Epstein-Barr virus (EBV), (b) monoclonal rearrangement of immunoglobulin and T cell receptor genes, and (c) rearrangement of the c-myc oncogene. Immunoglobulin and T cell receptor gene studies demonstrated that the tumor was of monoclonal B cell origin. Similar to cases of Burkitt's lymphoma unrelated to AIDS, there were DNA sequences in the lymphoma that hybridized to EBV-specific probes and demonstrated evidence of c-myc rearrangement. HTLV-III sequences were not detected in the malignant B cells. The pathogenesis of some B cell neoplasms in patients with the syndrome may involve transformation by EBV and deregulation of oncogene expression without direct infection of the malignant B cells by HTLV-III.

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THE ACQUIRED IMMUNODEFICIENCY syndrome (AIDS) is a transmissible disorder etiologically linked with infection by human T cell lymphotropic virus, type III (HTLV-III).[1–3] The major clinical manifestations of AIDS include opportunistic infections or opportunistic neoplasms, such as Kaposi's sarcoma and central nervous system lymphoma.[4] Recently, B cell lymphomas outside the central nervous system have been described in individuals with AIDS or among homosexual men epidemiologically at risk for the disorder.[5–9] We have studied the roles of HTLV-III, the Epstein-Barr virus (EBV), and the c-myc oncogene in the pathogenesis of a B cell lymphoma in a man with AIDS. Immunoglobulin plus T cell receptor gene studies were used to confirm monoclonal B cell origin. Our findings suggest that EBV potentiates the development of B cell neoplasia, while HTLV-III sets the immunosuppressive milieu but does not appear to infect the malignant B cells directly. Rearrangement of the c-myc oncogene occurred in this case similar to rearrangement in Burkitt's lymphoma unrelated to AIDS.

MATERIALS AND METHODS

The patient was a 43-year-old Hispanic man whose risk factor for AIDS was 20 years of multiple homosexual contacts. He was diagnosed as having AIDS by biopsy of a cutaneous lesion that revealed Kaposi's sarcoma. His major clinical manifestations of AIDS included unexplained fevers, diarrhea, and progressive dementia. No enteric pathogens or parasites were identified in his stool and an upper and lower endoscopy revealed Kaposi's sarcoma. The etiology of his dementia was not determined. Lumbar puncture revealed normal glucose of 58 mg/dL, elevated protein of 78 mg/dL, and 10 mononuclear cells per microliter. CT scan of the brain showed generalized atrophy, cultures of cerebrospinal fluid for bacteria, fungi, and mycobacteria were negative, and cytology was unremarkable. The patient died and an autopsy was performed. Sera were collected at multiple times during the course of his illness, and tissues were immediately cryopreserved at the time of death.

Complete blood count, total lymphocyte count, and T cell subsets were performed as previously described.[10] Antibodies to EBV early antigen, nuclear antigen, and viral capsid antigen were measured as previously described.[11] Antibodies to HTLV-III were measured by an enzyme-linked immunosorbent assay and Western electroblot technique.[12] Multiple sections of the B cell lymphoma were studied for EBV nuclear antigen using fluorescent techniques.[3] For immunoglobulin and T cell receptor gene studies, DNA was prepared from a biopsy specimen of the lymphoma, digested with the appropriate restriction enzyme and examined by Southern blot hybridization with the corresponding radiolabeled probe using standard methods, as previously described.[13] Immunoglobulin gene probes were as described by Korsmeyer et al[14]—J_H (2.4-kilobase [kb] sau3a fragment), C_K (2.5-kb *Eco*RI fragment), $C\ \lambda$ (0.8-kb *Bam*HI–*Hind*III fragment). T cell receptor gene probes were C_β (0.6-kb *Bgl*II–*Eco*RV fragment of YT35)[15] and $C\ \alpha$ (1.2-kb *Eco*RI fragment of clone V7d).[16]

DNA was also probed for sequences related to EBV, cytomegalovirus, and human T cell leukemia virus, types I, II, III, as well as for rearrangement of the c-myc oncogene.[3,16,17] The ontogeny of the tumor was studied by immunofluorescent staining of tissue sections using specific antisera to the immunoglobulin kappa and lambda light chains.

RESULTS

Autopsy revealed extensive replacement of lymph nodes by large cells with vesicular nuclei and nucleoli. Masses containing similar cell types were distributed throughout the gastrointestinal tract, liver, and spleen. The brain showed generalized loss of gray and white matter with inclusion bodies consistent with cytomegalovirus. No lymphoma was found in the brain. On light microscopy, the histology of the tumor was consistent with a diffuse large-cell lymphoma. Immunofluorescent studies for immunoglobulin light chains revealed a predominance of lambda light chains, suggesting a monoclonal origin. Immunoglobulin gene studies performed on DNA prepared from the lymphoma (Fig 1)

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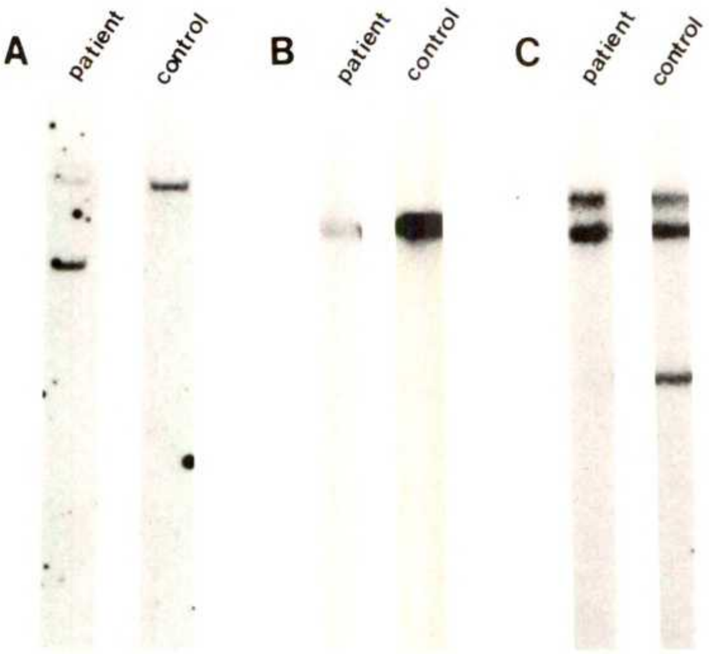

Fig 1. Detection of immunoglobulin gene rearrangement by Southern hybridization. DNA from a biopsy specimen of the patient's lymphoma was examined by Southern blot hybridization using immunoglobulin gene probes as described in Materials and Methods. In all three panels (A through C) the lymphoma DNA is shown in the first lane, with control DNA (from a normal unrelated individual) in the germline configuration in the second lane. Dashes indicate the expected position of germline bands.[14] (A) Immunoglobulin heavy chain locus (*Bam*HI/J_{H2} probe). A prominent rearranged band is evident at the lower arrowhead, consistent with a monoclonal B cell population. The absence of a second band of equal intensity suggests deletion of the second heavy chain locus in these cells. The faint persistent germline band may be due to a small amount of residual normal tissue in the biopsy specimen. The upper arrowhead marks a second faint rearranged band. This may represent incomplete detection of a rearrangement at the second heavy chain locus. We cannot exclude a rearrangement due to a second, smaller monoclonal cell population (a "biclonal" lymphoma). (B) Kappa light chain locus (*Bam*HI/C_κ). Only a faint germline band is evident in the lymphoma DNA. This is most consistent with deletion of both kappa gene loci, with the faint band due to residual normal tissue in the biopsy specimen, as above. (C) Lambda light chain focus (*Eco*RI/C λ). The absence of one germline band from the lymphoma DNA is evident. This pattern could be consistent with deletion or rearrangement of one lambda light chain gene. However, since nonlymphoid DNA from this patient was not examined, we cannot exclude the presence of a restriction fragment length polymorphism at the lambda light chain locus[14] as the explanation for this pattern.

revealed evidence for rearrangement at the heavy chain locus and probable deletion of both kappa light chain loci. Examination of the T cell receptor alpha and beta chain loci revealed exclusively the germline configuration with no evidence for clonal rearrangement (data not shown). This pattern is most consistent with monoclonal proliferation of B cell origin and of lambda light chain type. These results were in agreement with the immunofluorescent studies.

Serologic studies indicated antibodies to EBV early, nuclear, and capsid antigens, cytomegalovirus, and HTLV-III (Table 1). Antibodies to HTLV types I or II were not present. The tumor tissue expressed EBV nuclear antigen detected by immunofluorescence. Southern analysis revealed sequences related to EBV in the B cell lymphoma (Fig 2) but no hybridizing sequences using probes for cytomegalovirus, HTLV-III, HTLV-I, and HTLV-II. Rearrangement near the c-myc gene was also detected by Southern analysis (Fig 3).

DISCUSSION

B cell lymphomas occur with increased frequency in a variety of hosts with inherited or acquired deficiencies of the cellular immune system.[18-21] Considerable epidemiologic and molecular biologic data suggest that some of these B cell lymphomas are associated with the presence of circular EBV genome and possibly integration of EBV into host genome.[11,19] Polyclonal B cell hyperplasia is frequently found in lymph nodes of patients with AIDS or AIDS-related complex.[8] In some cases, there appears to be an initial

Table 1. Laboratory Studies

Serology	
EBV-EA	1:80
EBV-VCA	1:640
EBNA	1:160
CMV-CF	1:128
HTLV-III-ELISA	+
HTLV-III-Western blot	+
Molecular Studies on Lymphoma DNA	
EBV	+
HTLV-III	−
Immunoglobulin rearrangement	+
T cell antigen receptor rearrangement	−

EBV, Epstein-Barr virus; EA, early antigen; VCA, viral capsid antigen; EBNA, EBV-nuclear antigen; CMV-CF, cytomegalovirus complement fixation; HTLV-III-ELISA, enzyme-linked immunosorbent assays.

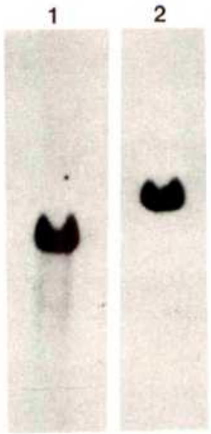

Fig 2. Detection of EBV DNA in lymphoma tissue by Southern hybridization. DNA (20 μg) extracted from lymphoma tissue obtained at autopsy was cleaved with *Bam*HI, subjected to electrophoresis (1.5 V/cm, 18 hours) through a 0.75% agarose gel and transferred to a nitrocellulose sheet. This gel replica was hybridized to PKD-52, a *Bam*W fragment of EBV in pBR322 (a gift from E. Kieff, University of Chicago, Chicago). Lane 1, 20 μg lymphoma DNA, lane 2, 30 pg pBR322 was added to 20 μg lymphocyte DNA from an EBV-seronegative control, equivalent to ten copies of viral DNA per cell. The hybridization in lane 2 is with pBR322 and not with EBV, as shown by different location of the hybridization band compared with lane 1.

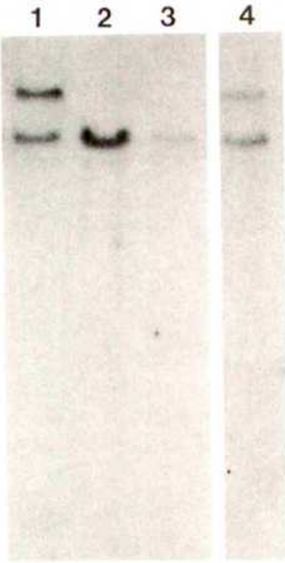

Fig 3. Detection of rearranged c-myc gene by Southern hybridization. Lymphoma tissue DNA (20 μg) was cleaved with *Eco*RI, the DNA fragments were separated by electrophoresis (1.7 V/cm, 18 hours) through a 0.75% agarose gel. The DNA was then transferred to cellulose nitrate under partial vacuum, fixed, and hybridized with ^{32}P-labeled 3rd exon of human c-myc (Hu 3′ myc, 1.2 kb, RI-*Cla*, 3rd exon) in pBR327 (Taub) (a gift from P. Leder, Harvard Medical School, Boston). Lane 1, Raji (Burkitt's lymphoma with detectable rearrangement of c-myc) cell DNA; lane 2, P3HR1 (Burkitt's lymphoma without detectable rearrangement of c-myc) cell DNA; lane 3, lymph node DNA from a fatal EBV infection in a patient with X-linked lymphoproliferative syndrome with a polyclonal, karyotypically normal EBV-induced B cell proliferation; lane 4, lymphoma tissue DNA from the AIDS patient.

polyclonal activation of B cells, followed by emergence of a monoclonal neoplasm. HTLV-III is the primary etiologic agent in AIDS.[1-3] The T4 antigen, or a closely related membrane antigen, appears to be necessary for entry and permissive infection of HTLV-III in T lymphocytes.[22,23] Because some activated B cells express the T4 antigen and, in vitro, HTLV-III can infect T4-bearing lymphoblastoid cell lines transformed by EBV, it is possible that HTLV-III could directly infect in vivo activated B cells and play a primary pathogenetic role in the development of B cell neoplasia in AIDS.[23] Our study of this case argues against such a model. There were no sequences related to HTLV-III in the B cell lymphoma, despite clear serologic evidence of HTLV-III infection in the host. A more likely model, based on our study, would be the emergence of an EBV-transformed B cell clone resulting from impaired cellular "immunologic surveillance" due to infection of T cells by HTLV-III.

Serologic studies in AIDS demonstrate high titers of antibodies to EBV antigens, suggesting reactivation of this virus in some patients.[5] Previous case reports have indicated chromosomal translocations t(8:14) and t(8:22) in B cell lymphoma in AIDS.[6,7] Such translocations occur in endemic (African) and nonendemic Burkitt's lymphoma.[24-26] Karyotype was not done in our case, but DNA rearrangement near the c-myc gene was detected. The c-myc oncogene is located on chromosome 8 and is rearranged in Burkitt's lymphoma unrelated to AIDS.[7] The pathogenesis of some B cell lymphoma in AIDS may involve initial polyclonal expansion of the B cell population due to reactivation of EBV with subsequent emergence of a transformed monoclonal population characterized by chromosomal rearrangement and disordered regulation of the c-myc oncogene. The emergence of the transformed clone may be facilitated by HTLV-III infection and impaired immune surveillance.

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The Mutual Relationship Between the Two Molecular Forms of the Major Fibrinolysis Inhibitor Alpha-2-Antiplasmin in Blood

By C. Kluft, P. Los, A.F.H. Jie, V.W.M. van Hinsbergh, E. Vellenga, J. Jespersen, and Ch.P. Henny

Alpha-2-antiplasmin, a major inhibitor of fibrinolysis, is synthesized in the liver and occurs in blood in two molecular forms: a very active plasminogen-binding (PB) form and a less active nonplasminogen-binding (NPB) form. This study investigates the origin and mutual relationship of these two forms in vivo and in vitro. Despite wide variation in plasma concentration of the inhibitor (16% to 138%), the ratio between the two forms in vivo was found to be, in the main, constant among healthy volunteers, heterozygotes for a congenital deficiency of alpha-2-antiplasmin, and patients with a stable liver cirrhosis: PB/NPB = 2.41 ± 0.34 (SD). Resynthesis after depletion or increased synthesis in the acute-phase reaction showed a specific increase of the PB form of the molecule in blood after discontinuation of L-asparaginase or streptokinase therapy and after myocardial infarction. In vitro studies demonstrated that only the PB form was present after one day in the culture medium of the human cell line Hep G2, while the NPB form appeared after 11 days. Clearance after inhibition of synthesis by L-asparaginase therapy revealed a more rapid decrease in the PB form relative to the NPB form in blood, demonstrated by a change in the PB-NPB ratio from 2.86 ± 0.55 to 1.74 ± 0.24 (mean of 6, SD). An apparently spontaneous first order conversion from the PB to NPB form, with an apparent half-life of about eight days, was demonstrated at 37 °C in plasma and serum in vitro. The conversion was found to be temperature dependent and uninfluenced by the fibrinolytic components fibrinogen, fibrin, and plasminogen. Additions of a variety of enzymes or inhibitors did not interfere with the process. These results demonstrate that the PB form of alpha-2-antiplasmin is produced by the liver and that the NPB form is formed in the circulation.
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ALPHA-2-ANTIPLASMIN is a plasma glycoprotein with an apparent molecular weight (mol wt) of 68,000.[1,2] It occurs in blood in two molecular forms: plasminogen binding (PB) and nonplasminogen binding (NPB).[3] The PB form inhibits plasmin-activated fibrinolysis by rapid formation of a stoichiometric plasmin complex.[2,4] It further interacts with fibrinolysis by forming a reversible complex with plasminogen and by covalent binding to fibrin during coagulation caused by the action of the fibrin stabilizing factor (factor XIII).[5,6] The NPB form reacts much more slowly with plasmin,[3] does not bind to plasminogen, and is not significantly cross-linked to fibrin.[7] Alpha-2-antiplasmin plays a critical role in the in vivo regulation of fibrinolysis, as shown by the hemorrhagic diathesis found in congenitally deficient patients[8-11] as well as in some heterozygotes.[9,10] This role is attributed to the PB form; the relevance of the NPB form is unknown.

The site of synthesis of alpha-2-antiplasmin appears to be the liver, as recently supported by demonstration of synthesis in an established liver cell line[12] and in rat hepatocytes.[13] It is unknown, however, which form of alpha-2-antiplasmin is synthesized and the mutual relationship of the two forms is unclear. Circumstantial evidence has been provided that the NPB form can be obtained from the PB form in vitro[14] and in vivo.[15]

To determine the origin and mutual relationship of the two forms of alpha-2-antiplasmin, we studied their occurrence and behavior under various conditions in blood and in culture media of a liver cell line with a recently devised assay for both forms.[16] The study provides data that demonstrates the synthesis of the PB form and the formation of the NPB form in the circulation.

MATERIALS AND METHODS

Materials. Unless otherwise specified, reagents were of analytical grade and obtained from Merck, Darmstadt, FRG. "Agarose for electrophoresis" (lot No. 33006) and iodoacetamide were obtained from BDH Chemicals Ltd, Poole, England. Carbowax 6000 was from Fluka AG, Buchs, Switzerland. Antiserum raised in rabbits against human alpha-2-antiplasmin was a gift from Dr I. Clemmensen, Copenhagen. Lys-plasminogen was prepared from human Cohn fraction III by affinity chromatography on lysine-agarose,[17] followed by gel filtration on Sephadex G-150. Trasylol (5880 KIU [kallikrein inactivator units]/mg) was a gift from Bayer AG, Wuppertal, FRG, through the courtesy of Dr E. Philipp. The synthetic tripeptide H-D-Val-Leu-Lys-pNA (S-2251) for plasmin was from AB Kabi, Stockholm.

Ellagic acid, actin was from Dade Diagnostics Inc, Miami. Dextran sulphate, sodium salt (mol wt 500,000) was obtained from Pharmacia Ltd, Uppsala, Sweden. Active Cls-esterase was prepared from outdated plasma according to Vroon et al[18] and Haines and Lepow.[19] Trypsin inhibitor from soybean type I-S, from turkey egg white type II-T and diisopropylphosphofluoridate (DFP) were from Sigma Chemical Co, St Louis. Lima bean trypsin inhibitor, ovoinhibitor, and ovomucoid were from Worthington Biochemical Corp, Freehold, NJ. Whole extracts of granula of polymorphonuclear granulocytes were prepared as described before.[20] Elastatinal, pepstatin, and chymostatin (see ref. 21) were gifts from Dr H. Umezawa, Microbiological Chemistry Research Foundation, Tokyo.

Plasma. Platelet-poor citrated human plasma and pooled plasma were prepared as described by Kluft et al[22]; serum was prepared by incubation of nonanticoagulated blood at 37 °C for four hours in plastic tubes before centrifugation. Factor XII- and factor XIII-deficient plasma were obtained from George King Bio-Medical Inc, Overland Park, Kansas. Plasma depleted in plasma urokinase

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was prepared by chromatography on column coupled antibodies to urokinase as described by Kluft et al.[23] Plasma depleted in plasminogen was prepared by chromatography on lysine-agarose (cf ref. 17); the depleted plasma showed no response on immunochemical assay for plasminogen (detection limit about 5%) and a residual activity of 1.6% in the streptokinase method of Friberger et al.[24]

Assays. Modified crossed immunoelectrophoresis with added lys-plasminogen was carried out as described by Kluft and Los.[16] Briefly, the 1% agarose gel for the first dimension in 0.03 mol/L buffer, pH 8.6, contained 1,000 KIU/mL Trasylol and 0.04 mg lys-plasminogen/per milliliter added to the agarose solution just before casting the gel. Before electrophoresis, 5 μL of plasma or serum, 2 μL of lys-plasminogen solution (2 mg/mL), and 1 μL of 10,000 KIU/mL Trasylol were sequentially and rapidly introduced into the punched well. The gel for the second dimension contained antiserum against alpha-2-antiplasmin. The immunoprecipitation peak surface at β-mobility represents the concentration of the PB form of alpha-2-antiplasmin, and at α-mobility, that of the NPB form. The antiserum had a comparable titer for both forms of alpha-2-antiplasmin as checked by assay of mixtures of the PB and NPB forms.[25]

The activity assay of alpha-2-antiplasmin, the immediate plasmin inhibition test, was performed as described by Kluft et al[9] and measured 1.00 PB + 0.14 NPB.[25] The PB and NPB concentrations can be calculated separately knowing the ratio PB-NPB from the modified crossed immuno-electrophoresis and the result of the immediate plasmin inhibition test using a standard (pooled plasma) with a known ratio and amount of inhibitor calibrated on active-site titrated plasmin.[26] Alternatively, PB and NPB concentrations can be determined directly by modified crossed immunoelectrophoresis in simultaneous runs with pooled normal plasma with known amounts of PB and NPB.[25]

Alpha-2-antiplasmin determined immunochemically by the technique of Laurell[27] was expressed in percentage of pooled normal plasma (1.07 μmol/L).

Changes in the conversion rate of plasma in vitro of the two forms of alpha-2-antiplasmin were assessed by incubation of the plasmas for 18 days at 37 °C and assay of the pattern of the modified crossed immunoelectrophoresis. Depleted or deficient plasmas were compared with starting plasma or pooled normal plasma. Effects of inhibitors (25 μL added to 250 μL plasma) were compared with buffer controls. The effects of Cls-esterase, granulocyte enzymes, dextran sulphate, and ellagic acid on the antiplasmin activity in the immediate plasmin inhibition test were studied after 15 minutes' incubation at 37 °C in 1:1 mixtures.

Volunteers and patients. Normal volunteers were from the institute. Heterozygotes with congenital alpha-2-antiplasmin deficiency were from a family described by Kluft et al.[9] Patients with advanced liver cirrhosis were in a stable period. The patient receiving local streptokinase for a thrombus was treated with a bolus injection of 5,000 units and a maintenance dose of 5,000 U/h for 24 hours. The dosage was increased to 10,000 U/h until the therapy was stopped after 97 hours. Samples obtained during and shortly after therapy were analyzed in the modified crossed immunoelectrophoresis assay in a special way. To account for the possible presence and interference by plasmin–antiplasmin complexes at β-mobility, an intermediate agarose gel layer containing antiserum against plasminogen[16] was introduced. In some samples, the precaution proved necessary. Three patients from a larger study[28] of patients with chest pain admitted to hospital with a diagnosis of myocardial infarction, later confirmed by electrocardiography and enzyme studies (AMI <48 hours), were further investigated after demonstration of a rise in activity in the immediate plasmin inhibition test.

Six patients with acute lymphocytic leukemia described by Vellenga et al[29] were studied before the start, at the end, and six to ten days after discontinuation of L-asparaginase intravenous therapy. The daily dosage varied from 5,500 to 7,496 U/m². The total duration of the therapy varied from seven to 17 days and resulted in reduction in the immediate plasmin inhibition test to 5% to 30% of normal, reverting to normal again after discontinuation.[29]

Informed consent was obtained.

Cell culture. The established cell line Hep G2 derived from a human liver tumor was obtained from Dr B.B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia) and is known to produce alpha-2-antiplasmin.[12] The cells were cultured at 37 °C in 25-cm² flasks containing 0.1 to 0.2 mL/cm² (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Irvine, Scotland), penicillin (100 IU/mL), and streptomycin (0.1 mg/mL) under a 5% CO_2/air atmosphere. For the experiments on alpha-2-antiplasmin, cells were grown to a density of 3 mg protein/25 cm². After two washings with DMEM, 2 mL of fresh medium were added to each flask. Media were harvested after one and eight days of incubation at 37 °C. During the incubation of 11 days, 1 mL of fresh medium was supplied on day 8. The media were centrifuged to remove cellular debris and stored at −20 °C. For the purpose of analysis, the medium was ten times concentrated on a Minicon concentrator B 15 (Amicon BV, Oosterhout, The Netherlands).

RESULTS

Steady state. As shown in Fig 1, the ratio PB-NPB is not significantly dependent on the synthesis rate of alpha-2-antiplasmin under steady state conditions, eg, in stable individuals. The data was obtained from apparently normal individuals, from heterozygotes for congenital alpha-2-antiplasmin deficiency with an approximately half normal plasma concentration, and from patients with liver cirrhosis with an apparently impaired synthesis of the inhibitor in a stable period. (It was recently demonstrated that no enhanced catabolism occurs in such patients.[30]) The total amount of alpha-2-antiplasmin (PB + NPB) was determined by Laurell immunoassay. The ratio PB-NPB in this group (Fig 1) was 2.41 ± 0.34 (SD), well in accord with experience in a larger group of 29 apparently healthy volunteers showing 2.33 ± 0.29 (SD).

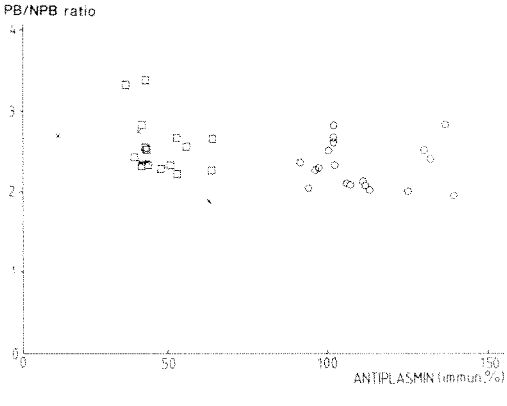

Fig 1. The ratio PB-NPB determined by the modified crossed immunoelectrophoresis technique for healthy volunteers (○; n = 19), patients with a stable liver cirrhosis (×; n = 5), and heterozygotes for congenital alpha-2-antiplasmin deficiency (□; n = 15). The total amount of alpha-2-antiplasmin is determined by the Laurell technique (abscissa).

Acute phase reaction. The alpha-2-antiplasmin activity as well as the PB and NPB forms were determined in three patients who showed an acute-phase reaction after myocardial infarction. During the acute phase, the increase in alpha-2-antiplasmin activity coincided with an increase in PB antiplasmin, while the NPB form remained virtually constant (Fig 2).

Streptokinase therapy. During streptokinase therapy of a patient (see Fig 3), alpha-2-antiplasmin was almost consumed by the active process. After treatment, the alpha-2-antiplasmin activity was restored to almost normal by resynthesis at the first blood sampling (indicated as "3" in the figure). At this particular time, as shown by the modified crossed immunoelectrophoresis pattern, the PB alpha-2-antiplasmin had almost exclusively reappeared.

L-asparaginase therapy. During L-asparaginase therapy, synthesis of alpha-2-antiplasmin was inhibited. A gradual, functional, and immunochemical lowering of the plasma concentration with a half-life of approximately five days was observed in six patients. At the discontinuation of therapy, alpha-2-antiplasmin was found to be 26% ± 9% of normal (immunoassay). A concomitant significant change was observed in the ratio of PB-NPB for all patients: from 2.86 ± 0.55 at the start to 1.74 ± 0.24 at the end of treatment. This indicates a selective reduction in PB alpha-2-antiplasmin. After treatment, resynthesis resulted in recovery of normal alpha-2-antiplasmin levels (92% ± 12%, after about eight days) and an increased PB-NPB ratio of 4.65 ± 1.32 (n = 6), indicating selective increases in PB alpha-2-antiplasmin.

Hep G2 cell culture. The hepatoma cell line Hep G2 produces alpha-2-antiplasmin[12] linearly in increasing amounts, with the incubation time as shown in Fig 4 by normal crossed immunoelectrophoresis. In the modified crossed immunoelectrophoresis, significant amounts of PB alpha-2-antiplasmin only were present at day 1, while on day 11 the NPB form had also appeared.

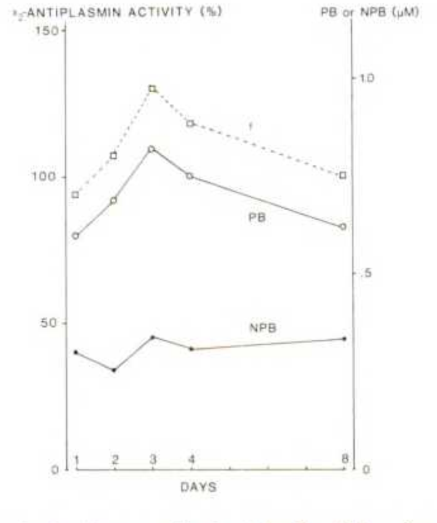

Fig 2. Acute-phase reaction in alpha-2-antiplasmin activity (f) in three patients with acute myocardial infarction. The PB and NPB forms are calculated from data from the modified crossed immunoelectrophoresis technique (PB-NPB ratio) and the activity assay (PB + 0.14 NPB) and expressed in μmol/L by comparison with normal plasma determined to contain 1.07 μmol/L alpha-2-antiplasmin in a PB-NPB ratio of 2.2.[25] The activity is determined by the immediate plasmin inhibition test and expressed in percentage of pooled normal plasma. The samples are collected at the indicated days after admission of the patient to hospital.

Incubations in vitro. Incubation of citrated pooled normal plasma in vitro was found to result in a slow decay of alpha-2-antiplasmin activity. Figure 5 shows that this pro-

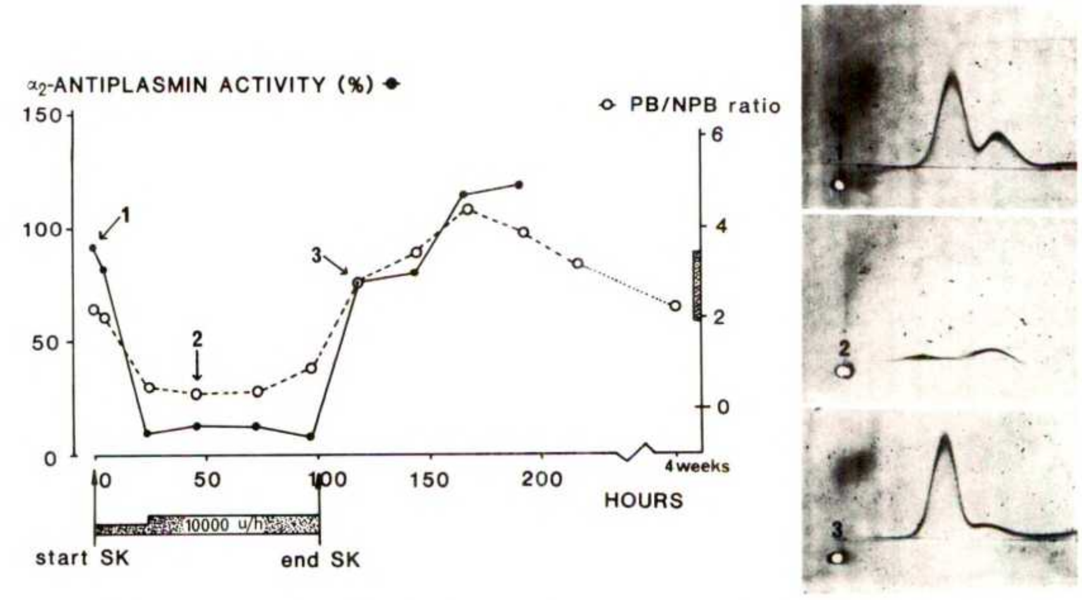

Fig 3. The two forms of alpha-2-antiplasmin during and after streptokinase therapy. The activity of alpha-2-antiplasmin is recorded by means of the immediate plasmin inhibition test and recorded in percentage of pooled normal plasma (ordinate). The PB-NPB ratio is determined by the modified crossed immunoelectrophoresis technique (normal range is indicated) and the gels for the indicated points 1, 2, and 3 are given in the insert.

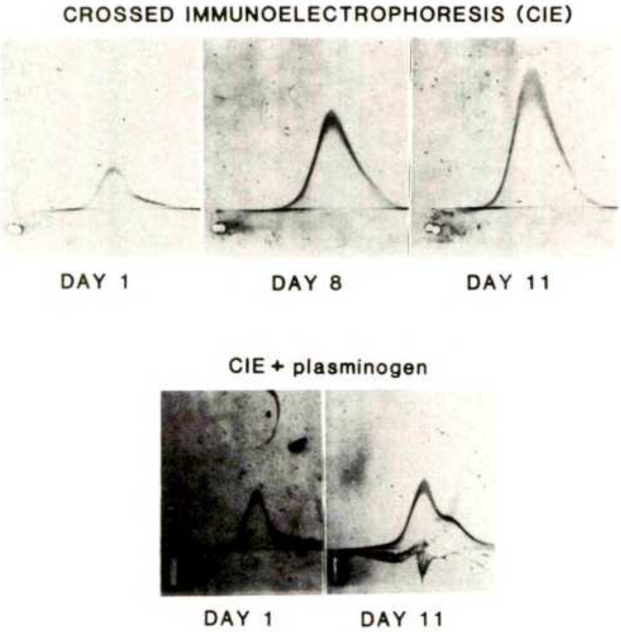

Fig 4. Production of alpha-2-antiplasmin in culture media of Hep G2 cells. Normal crossed immunoelectrophoresis shows an increased production with time (upper panel). Modified crossed immunoelectrophoresis (CIE + plasminogen) of medium (concentrated to comparable alpha-2-antiplasmin concentrations) of day 1 and day 11 are given in the lower panel.

cess exhibits individual variation that, for the initial rate, was estimated to show a range of ± 20% around the mean. As shown in Fig 6 for pooled normal plasma, the process is moderately temperature dependent, with an increase in rate of a factor of 2.4 and 1.9 for 0 °C to 25 °C and 25 °C to 37 °C, respectively.

As shown in the insert of Fig 6, the decay of activity is accompanied by a conversion from PB to NPB alpha-2-antiplasmin. Combining the activity assay and the modified crossed immunoelectrophoresis to determine PB and NPB separately (see Materials and Methods), it was demonstrated (as shown for pooled normal plasma in Fig 7) that the PB form shows a first-order decay with a half-life of eight days.

The conversion of PB to NPB on in vitro incubations was found to be uninfluenced by a number of circumstances. The

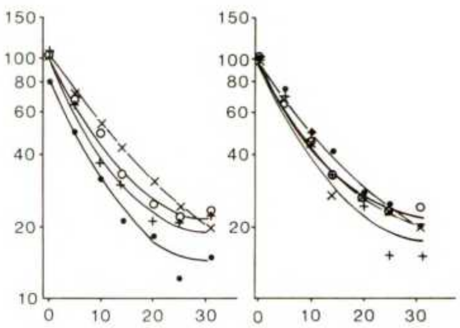

Fig 5. Decay of activity in the immediate plasmin inhibition test by incubation of citrated plasma (with azide) of eight apparently healthy volunteers for the indicated periods (abscissa) at 37 °C. The results of the inhibition assay are recorded in percentage of that of pooled plasma (ordinate, log scale). The activity decay shows the conversion of PB to NPB because of the reduced expression of NPB relative to PB (14%; ref. 25) in the activity assay.

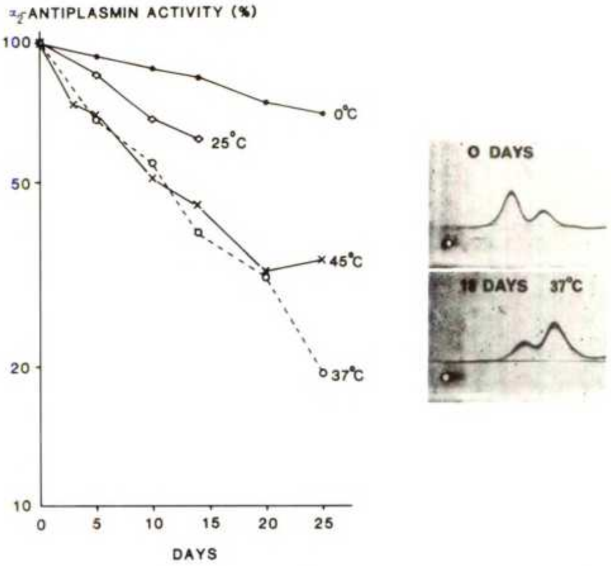

Fig 6. Decay of alpha-2-antiplasmin activity determined by the immediate plasmin inhibition assay (expressed in percentage of pooled plasma, ordinate, log scale) after incubation of citrated pooled plasma (with addition of azide) at various temperatures. The insert shows a typical example of the change from the PB to the NPB form during the incubation. (The exhibit gels are not run under identical conditions for the first dimension.)

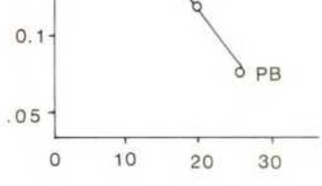

Fig 7. The conversion of PB to NPB in citrated pooled plasma by incubation at 37 °C for the indicated periods (abscissa). PB and NPB (log scale) are calculated from the following data from each sample: modified crossed immunoelectrophoresis gives PB/NPB; the immediate plasmin inhibition test gives PB + 0.14 NPB. Results were in accord with direct assay of PB in one run of the modified crossed immunoelectrophoresis for t = 0, 12, and 20 days.

conversion was no different in normal serum or plasma deficient in factor XII or factor XIII, or in plasma depleted in plasminogen (by sepharose-lysine[17]) or plasma urokinase (by immunoadsorption[23]). The conversion was not influenced by EDTA (1 mmol/L), by addition of granulocyte enzymes, by contact activation by either ellagic acid (1:1 with 10^{-4} mol/L) or dextran sulphate (1:1 with 25 μg/mL), or by addition of Cls-esterase (375 U/mL). The addition of the following inhibitors (highest final concentrations tested) did not prevent the conversion, as judged from the conversion after 18 days in the modified crossed immunoelectrophoresis: DFP (1 mmol/L), soya bean trypsin inhibitor (100 μg/mL), Trasylol (100 KIU/mL), benzamidine (100 μg/mL), lima bean trypsin inhibitor (100 μg/mL) ovomucoid (20 μg/mL), ovoinhibitor (100 μg/mL), elastatinal (1 μg/mL), pepstatin (1 μg/mL), chymostatin (1 μg/mL), iodoacetamide (100 mmol/L), β-mercaptoethanol (0.1 mmol/L), $HgCl_2$ (1 mmol/L), and $ZnCl_2$ (1 mmol/L).

DISCUSSION

Using modified crossed immunoelectrophoresis, the two forms of alpha-2-antiplasmin, PB and NPB, could be separately determined and both were present in all plasmas studied. The ratio between the forms was found to be rather constant among individuals, with a mean molar ratio of PB-NPB of 2:41. This was irrespective of the largely variable synthesis rate of alpha-2-antiplasmin, which resulted in corresponding plasma levels of total alpha-2-antiplasmin of 16% to 138% obtained by studying patients with stable liver cirrhosis, heterozygotes for alpha-2-antiplasmin, and normal individuals.

In situations where synthesis of new or additional alpha-2-antiplasmin was observed, such as after depletion by streptokinase or L-asparaginase therapy, or in acute-phase conditions after myocardial infarction, primarily the PB form of alpha-2-antiplasmin was found to be produced. Accordingly, in cultures of the hepatoma cell line Hep G2, the PB form was found to be initially produced, and only later was the NBP form also detected in the culture media. This demonstrates that the liver produces primarily (probably exclusively) the PB form of alpha-2-antiplasmin. As to the origin of the NPB forms of alpha-2-antiplasmin, it was demonstrated that the PB form apparently spontaneously converts to the NPB form in vitro. This was demonstrated in platelet-poor citrated plasma and indicates the involvement of plasma components; alternatively, the conversion involves an intrinsic property of alpha-2-antiplasmin. Assuming the exclusive synthesis of the PB form, the occurrence of the NPB form in vivo is evidence for its formation there as well. In addition, the data showing a more rapid disappearance of the PB form relative to the NPB form (decrease in the PB-NPB ratio) during therapy with L-asparaginase is compatible with and explained by an in vivo conversion of PB to NPB.

The data on conversion in vitro and the in vivo data agree when related to each other in a minimum hypothesis, which is also based on published data, as shown in Fig 8. First-order reactions are proposed for catabolism ($k2$, $k3$) of both forms and, as demonstrated in Fig 8, for the conversion of PB to NPB ($k1$). The recognition of the PB-NPB conversion implies that previous studies on the catabolism of injected radiolabeled alpha-2-antiplasmin do not report exclusively on $k2$. In the proposed model, the plasma half-life is represented by $\ln 2/(k2 + k3k1)$. This has been determined by Collen and Wiman[31] to give a half-life of 2.83 days on injecting healthy subjects with a preparation that, according to our evaluation of a similar one, mainly represents the PB form ($\geq$95%) (courtesy of Drs Knot and Collen; see ref.[30]). Using the additional information that the mean PB-NPB ratio in healthy subjects is 2.33, which represents $k3/k1$ during steady state, we can conclude that $k2$ in this model actually corresponds to a half-life of three days. In the model, then, $k1$ and $k3$ remain undetermined for the in vivo situation. If we assume the in vitro conversion of PB to NPB to be representative for the in vivo process, we can calculate that $k3$ has a value corresponding to a half-life of 3.4 days. These values are similar to the half-life of the plasma alpha-2-antiplasmin of 3.3 days, determined by Tamaki et al[32] by infusion of normal fresh plasma (PB + NPB mixture) in a patient with congenital deficiency of alpha-2-antiplasmin. It is not unrealistic to assume that both alpha-2-antiplasmin forms have closely similar catabolic rates, but definite proof awaits a direct determination of the catabolism of the NPB form. Alternatively, the assumption that the catabolic rates of both forms are identical results in a calculated $k1$ for the in vivo conversion comparable to a half-life of 7.2 days, which is close to the in vitro value of eight days.

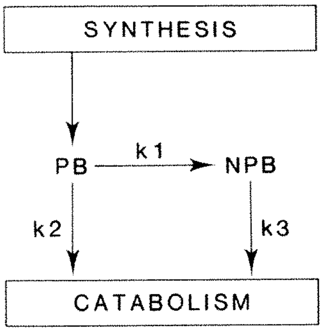

Fig 8. Proposed model for the mutual relationship between the PB and NPB forms of alpha-2-antiplasmin in the circulation. *k1*, *k2*, and *k3* represent first-order rate constants.

It is concluded that the model describes the quantitative aspects of the relationship between the two forms and implies no present need to assume other processes than the ones observed in vitro to describe the mutual relationsip between the two forms of alpha-2-antiplasmin. Further considerations are:

1. In the proposed model, the ratio PB-NPB is independent of the synthesis rate of alpha-2-antiplasmin in a steady state, as actually observed (Fig 1).
2. When synthesis is blocked, a relatively rapid decrease in the PB form in relation to the NPB form occurs, resulting in a shift in the PB-NPB ratio, as actually observed during asparaginase therapy.
3. The individual variation in the PB-NPB ratio (Fig 1) can only originate in a variation in $k1$, $k2$, or $k3$. According

to the data in Fig 6, it can originate in the variation in $k1$, which shows a comparable variability to that of the PB-NPB ratio.

The nature of the conversion process is not elucidated by this study, but it has been demonstrated that it concerns a temperature-dependent process in vitro, with a more moderate temperature dependence than enzymatic reactions ($Q_{10} = 2$ to 4). The recent report of Sasaki et al[33] that a C-terminal peptide has plasminogen-binding properties (cf ref. 34) would suggest the possibility of splitting off such a peptide. Our attempts to inhibit the conversion by a spectrum of protease inhibitors (directed to various serine and thiol proteases) was unsuccessful, and a number of proteases tested (added or generated in the plasma) were incapable of achieving or enhancing the conversion. It is significant that our experiments practically exclude the involvement of the known hemostasis components: fibrinogen, fibrin, factor XIII, plasminogen, plasma prourokinase, factor XII and its related processes. This strongly suggests the involvement of a previously unknown component or of an intrinsic property of the alpha-2-antiplasmin; the latter may be relatively less likely in view of the individual variation in the conversion (Fig 5).

The significance of the conversion of PB to NPB alpha-2-antiplasmin and that of the NPB form has still to be established. It appears that the NPB form represents partial inactivation of the alpha-2-antiplasmin as it is a much less reactive inhibitor with a substantially less avid plasmin inhibition and does not participate in plasminogen binding and not significantly in binding by the fibrin stabilizing factor (factor XIII) to fibrin. One of the important mechanisms to direct fibrinolytic processes or actions of plasminogen activators to fibrin lies in the particular features of the PB form of alpha-2-antiplasmin.

Because of the low spontaneous velocity of the conversion process, the conversion can only be involved in slow processes. This might be the case in setting free fibrinolysis in aging thrombi, in cavities with a slow flow, such as the synovium or cystes, and in parts of the extracellular space; however, the conversion may be enhanced, possibly locally.

Discovery of the mechanism, or hypothetical factors or cofactors, in the conversion process and manipulation of the conversion by these or related components might present an intriguing possibility for fibrinolytic therapy and might help to establish the importance of the process, especially if it proves to be liable to local variation.

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Iron Metabolism in the Belgrade Rat

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Iron metabolism in the Belgrade rat was examined in the intact animal and in the reticulocyte suspensions. The plasma iron turnover was increased. However, when allowance was made for the effect of the elevated plasma iron concentration, erythroid marrow capacity for iron uptake was at basal levels. Numbers of erythroid cells in marrow and spleen measured by the radioiron dilution technique were increased. Thus iron uptake was not proportionate to the erythroid hyperplasia in the b/b rat, despite a more than adequate plasma iron supply. This relative deficiency in iron uptake was reflected in a severe microcytosis and elevated red cell protoporphyrin.

Reticulocyte incubation studies demonstrated an unimpaired uptake of the transferrin–iron–receptor complex but a marked reduction in iron accumulation. The diferric transferrin molecule, when it did give up iron within the cell, released both of its iron atoms so that only apotransferrin was returned to the media. In contrast to the nearly complete release of iron within the normal reticulocyte, the major portion of iron taken up by the Belgrade reticulocyte was returned to the plasma. The release mechanism that can be impaired in iron-deficient reticulocytes by EDTA or cadmium was shown to be affected by lower concentrations of these substances in the Belgrade reticulocyte. It is concluded that the Belgrade rat has an abnormality of iron release within the absorptive vacuole that is responsible for a state of intracellular iron deficiency, involving the erythron and other body tissues.

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THE ANEMIA of the Belgrade rat is an autosomal recessive trait, determined by a radiation-induced mutation.[1] It is characterized morphologically by red cell hypochromia, microcytosis, and reticulocytosis.[2] There is a modest increase in total iron-binding capacity of the serum with a proportionately greater increase in the serum iron concentration.[2] Despite the hyperferremia, however, there is an absence of stainable iron in the body tissues and a lack of sideroblasts in the marrow.[2] Studies in a small number of animals suggest that iron utilization for hemoglobin synthesis is reduced.[3] Parenteral iron treatment fails to cure the anemia, even though it results in a gross iron overload of spleen and liver [2]

More detailed studies of the behavior of immature erythroid cells have shown an impaired iron uptake of transferrin iron.[4] Globin chain synthesis in reticulocytes, as measured by the incorporation of 3H-L-leucine in vitro, is decreased but balanced.[4] Incubation of reticulocytes with exogenous hemin partially corrects the defect in globin synthesis but has little effect on iron uptake.[5] When reticulocytes are exposed to ^{59}Fe, ^{125}I-labeled transferrin, there is an unimpaired uptake of transferrin but a decreased uptake of iron.[6,7] The examination of ^{125}I-labeled cell membrane in acrylamide gel does not disclose an obvious difference in receptor sites for transferrin.[7] In summary, past studies have shown a block in the acquisition of iron by body tissues, the nature of which has not been precisely defined. The purpose of this study was to provide more quantitative dimensions to the iron uptake abnormality and to define the nature of the abnormality.

MATERIALS AND METHODS

Anemic Belgrade laboratory male rats (b/b) of a mixed genetic background were mated with normal female Long Evans rats to provide obligatory heterozygotes. These heterozygotes were intercrossed to provide anemic (b/b) and nonanemic (−/b, −/−) control animals for studies of erythropoiesis. Because it was not possible to distinguish between −/b carriers and −/− normal homozygotes, the expression −/? will be used to denote the nonanemic control animals. Although heterozygous (+/b) litter mates are not normal from the genotypic point of view, they are normal phenotypically Other workers and ourselves have not been able to demonstrate any phenotypic differences between +/+ and +/b animals In view of the high reticulocyte count of b/b animals, normal animals rendered iron deficient by bleeding and low iron diet with comparable reticulocyte counts were used in incubation studies as a control.

Ferrokinetic studies. Plasma iron turnover measurements were performed by injecting radioiron as $FeSO_4$ intravenously into control animals and radioiron bound to normal plasma into homozygous animals [8] In the latter instance, the iron saturation of transferrin was set at that present in vivo, usually about 80%. Five samples of blood, each 0.2 mL, were withdrawn from the tail vein over the anticipated T½ disappearance time, and additional blood was drawn with the last sample for determination of plasma iron concentration and total iron-binding capacity. The plasma iron turnover (mg of iron/dL whole blood/d) was calculated according to the formula:

$$\frac{\text{PIT} = \text{plasma iron } (\mu/\text{dL}) \times \text{plasmatocrit}}{\text{T}^{1\!/\!2}\,(\text{min}) \times 100}.$$

In addition, a correction was made for the effect of plasma iron concentration on plasma iron turnover.[9] In this correction, the PIT is converted to that which would be found at 100% transferrin saturation. The basis for this correction is the molecular advantage of 11.6 in iron delivery by diferric transferrin as compared with monoferric transferrin in the rat. The formula is:

$$\text{PIT at 100\% sat} = \text{PIT(s)} \times \frac{\frac{200}{s} + 9.6}{11.6},$$

where s = transferrin iron saturation

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Erythroid cell number. Total marrow cellularity was determined in rats sacrificed six to eight hours after the injection of radioiron at which time plasma activity had decreased to <20% of the original level. Animals were exsanguinated and perfused with saline. Total marrow cellularity was determined in these animals by relating the cell count and radioactivity of an aliquot of marrow to the radioactivity of the total skeleton as previously described.[10] Immediately after death, one humerus was removed and its activity was determined. Then its marrow was flushed from the open end, using a syringe filled with Hanks' buffered saline solution. The collected cells were refluxed with a syringe and 27-gauge needle until a uniform cell suspension was obtained. The number of cells and radioactivity of this suspension was determined. Then the suspension was centrifuged at 400 g and the supernate removed for determination of radioactivity. The animal carcass minus the viscera was then autoclaved and all bones removed for determination of total skeletal activity.

Marrow cellularity (MC) was determined in two ways. The first method calculated total erythroid cells of the marrow from the number of erythroid cells counted in the aliquot and the total activity of the aliquot (cells and supernate) as compared with skeletal activity. The second method calculated total erythroid marrow cellularity from the number of erythroid cells in the aliquot and the radioactivity of centrifuged cells alone *v* that of the total skeleton. If activity in the supernate of the aliquot represented shed cytoplasm but the cells were still countable, the first method would be the appropriate one. If the activity in the supernate of the aliquot represented broken cells that could not be counted, the second method would be proper. These two calculations are assumed to represent minimum and maximum figures, the true value lying somewhere between. The formulas used for these two calculations were as follows:

$$\text{MC (minimal)} = \text{aliquot erythroid cells} \times \frac{\text{total skeletal activity}}{\text{total cell suspension activity}}$$

$$\text{MC (maximal)} = \text{aliquot erythroid cells} \times \frac{\text{total skeletal activity}}{\text{aliquot cell button activity}}$$

In addition, at the time the animal was killed, the spleen was removed and weighed and activity was determined. An aliquot of spleen was then weighed and torn apart so as to release its cell content into Hanks' solution. This cell suspension was centrifuged, the supernate removed, and plasma substituted. It was then diluted to a known volume and shaken to a uniform suspension, and cells were counted. By relating the aliquot to the total spleen through weight ratio, total cell count was calculated.

$$\text{Splenic erythroid cells} = \text{splenic aliquot erythroid cells} \times \frac{\text{splenic weight}}{\text{aliquot weight}}.$$

Reticulocyte assay. Reticulocyte-rich blood (reticulocyte count 28% to 38%) was obtained from Belgrade and genetically normal iron-deficient rats. The cells were washed and preincubated in Hanks' balanced salt solution for 15 minutes to deplete the cells of prebound transferrin.[9] Then cells were incubated in Hanks' buffer with the transferrin preparations labeled with ^{55}Fe and ^{125}I or ^{59}Fe and ^{55}Fe (see Results) in Hanks' buffer. In chaser experiments, the cells were incubated for ten minutes at 37 °C with the tagged transferrin and 11 μg of cold diferric transferrin iron was added to start the chase. Populations of blood cells from iron-deficient control rats and Belgrade rats with the same reticulocyte count (28% to 33%) were prepared. Otherwise, the incubation mixture consisted of a transferrin concentration 1.0 mg/mL, dissolved in Hanks' balanced salt solution, pH 7.4, hematocrit 20% to 25%. A typical total volume for a single uptake evaluation was 0.72 ml.

Transferrin preparation. Purification and iodination of rat diferic transferrin with ^{125}I were done as described elsewhere.[11] Transferrin was converted to apotransferrin by using desferrioxamine.[12] For the preparation of di [^{59}Fe] ferric transferrin, 30 μCi of ^{59}Fe (13 to 22 μCi/μg of iron, dissolved in 0.5 mol/L HCl) (1 Ci = 3.7 × 10^6 Bq), was added to 50 mg of ^{125}I-labeled apotransferrin (100 μCi of ^{125}I) dissolved in 5 mL of 0.05 mol/L Tris HCl, pH 8.0/0.01 mol/L $NaHCO_2$. This was followed by the addition of sufficient iron as ferrous ammonium sulfate (273 μg of iron per milliliter in 0.01 mol/L HCl) to saturate exactly the free iron-binding sites present in the transferrin solution. This was achieved by a spectrophotometric titration technique in which the increase in color development at = 465 nm was observed.[12] At the point of saturation, the A465-A280 ratio of the transferrin solution was 0.046, characteristic of the diferric state of the transferrin molecule.[11] Monoferric transferrin was prepared by column chromatography on Sephadex G50 at pH 5.1. Double-labeling of the purified transferrin was achieved by first labeling all sites with ^{59}Fe, removing the iron from the acid labile site by chromatography at pH 5.1, and then saturating the open sites with $^{55}FeSO_4$.[11] The preparation had a specificity of site binding of more than 80%. No site-to-site exchange of isotope occurred when stored at 4 °C in 1 mmol/L bicarbonate-buffered saline (pH 7.4) for at least three weeks, as shown by repeating the Sephadex G-50 separation.[8] In addition, the incubation of the selectively labeled diferric transferrin preparation in Hanks' buffer at 37 °C for two hours without or with washed reticulocytes failed to show any scrambling of the two isotopes.

Miscellaneous methods. Hemoglobin was determined by the cyanmethemoglobin method, hematocrit by the micromethod, and red cell count was done manually. Plasma iron and iron-binding capacity were determined according to standard methods. Red cell protoporphyrin was determined spectrophotometrically.[13] Nonheme iron analyses were carried out by wet ashing and colormetric analysis, as described in Bothwell et al.[14] ^{59}Fe was counted in a gamma counter, whereas samples for ^{55}Fe analysis were wet ashed and counted in a scintillation counter.[15] When ^{59}Fe along with ^{125}I were used, both isotopes were measured without extraction in an autogamma scintillation spectrometer (Packard, Model 5330). Simultaneous measurement of ^{59}Fe and ^{55}Fe activity was carried out after acid digestion, using the method of Eakins and Brown, in a liquid scintillation counter (Packard, Model 2405).[15] Isolectric focusing in polyacrylamide gel was carried out as described elsewhere.[11] The methods of incubation, washing, wet ashing of the cells, counting radioactivity, chemical iron determination, and statistical evaluation were the same as previously described by this laboratory.[8,11,12] Variations are expressed as ± 1 SD.

Reagents for in vitro studies. Ferrous ammonium sulfate [$(NH_4)\ Fe(SO_4) \times 6H_2O$] was purchased from Baker Chemical Co, Phillipsburg, NY. Disodium EDTA and cadmium chloride ($Cd\ Cl_2 \times 2\frac{1}{2}\ H_2O$) were obtained from Sigma Chemical Co, St Louis, and from Analar BDH Chemicals Ltd, Poole, England, respectively. A 0.1 mol/L EDTA stock solution in saline was prepared and adjusted to pH 7.4 by the addition of 0.1 mol/L HCl. A 0.115 mol/L Cd CL_2 stock solution in pH 2.0 saline was prepared and stored at this low pH and neutralized in the experimental mixture by the addition of 0.5 mol/L HEPES buffer (pH 7.4).

RESULTS

Studies of erythropoiesis. Hematologic data on four adult homozygous (b/b) animals and four adult control litter mates, either heterozygous or normal (−/?), reared under

Table 1. Erythropoiesis in the Belgrade Rat

Items	Homozygous 1	Homozygous 2	Homozygous 3	Homozygous Average	Heterozygous or Normal 1	Heterozygous or Normal 2	Heterozygous or Normal 3	Heterozygous or Normal 4	Heterozygous or Normal Average
Cellularity									
BM Eryth (%)	45	64	43	51	30	37	44	43	38
BM Eryth (× 10^9/kg)	9 5/20 8	11.5/19.3	9.5/15.8	10.2/18.6	4.4/6.7	3.9/5.9	4.6/7.0	4.27/6 5	4.3/6.5*
Spleen Eryth (%)	71	39	45	52	13	10	.05	23	13
Spleen Eryth (× 10^9/kg)	14 7	3.6	5 9	8.1	.01	.01	.01	.05	02
Total Eryth (× 10^8/kg)	24/35	15/23	15/22	18/26.7	4.4/6.7	3.9/5 9	4 6/7.0	4 2/6.5	4.27/6 52*
Hct (%)	35	34	32	34	41	36	37	38	38
PI (μg/dL)	478	595	426	500	401	285	278	416	345*
Tf sat (%)	80	81	63	75	51	42	43	63	50*
PIT (mg/dL wb/day)	2.4	2 8	2.1	2 4	1 8	2.6	1.9	1.9	2.0*
PIT (100% sat)	2.5	2.9	2.3	2.6	2 5	3.2	2.3	2 1	2 5*

*Significance of $P <$ 05.

identical conditions were determined. Their respective mean hemoglobin concentration was 7.5 and 11.8 g/dL, mean cell volumes 34 and 63.4, and mean corpuscular hemoglobin concentration 19.0 and 27.8. Thus each b/b red cell had 0.37 the amount of hemoglobin as compared with the –/? red cell. Protoporphyrin values on four homozygotes were elevated to a mean value of 176 ± 74 as compared with a control value of 28 ± 6 μg/dL RBC. Liver and spleen iron content was lower in b/b animals than in controls, ie, 2.6 ± 2.0 *v* 5.5 ± 1.4 mg of iron. Despite this evidence of iron deficiency in circulating red cells and other tissues, plasma iron and transferrin saturation of the b/b animals (Table 1) were elevated significantly above those of control animals.

Quantitative measurements of erythroid cellularity (Table 1) showed an increase of between two and three times controls in the nucleated red cell precursors within the skeleton and a still greater increase in nucleated cells in the enlarged spleen of the homozygous rats. The total number of erythroid cells (spleen and marrow) of b/b animals was increased to about four times that of –/? rats. While the two methods of calculating total erythroid precurser cellularity gave different values, the comparison between homozygote b/b and –/? was almost identical, ie, 4.2 *v* 4.1 × basal.

The plasma iron turnover of homozygous animals averaged 2.5 mg/dL per whole blood per day as compared with 2.0 mg in control animals. However, if a correction was made for the effect of the higher plasma iron concentration in the homozygous animals,[9] the calculated plasma iron turnover of the two groups of animals at a transferrin saturation of 100% showed essentially no difference, ie, 2.6 and 2.5 mg iron per deciliter of whole blood. Thus, despite a far greater number of red cell precursors, a better iron supply, and a lower hemoglobin, the homozygous animals showed no increase in erythroid iron uptake. Red cell utilization of radioiron was similar in the two groups, ie, 81 ± 11 in the b/b *v* 86 ± 7 in the –/? animals.

Reticulocyte iron uptake and release. Further studies were directed more specifically at the defective reticulocyte

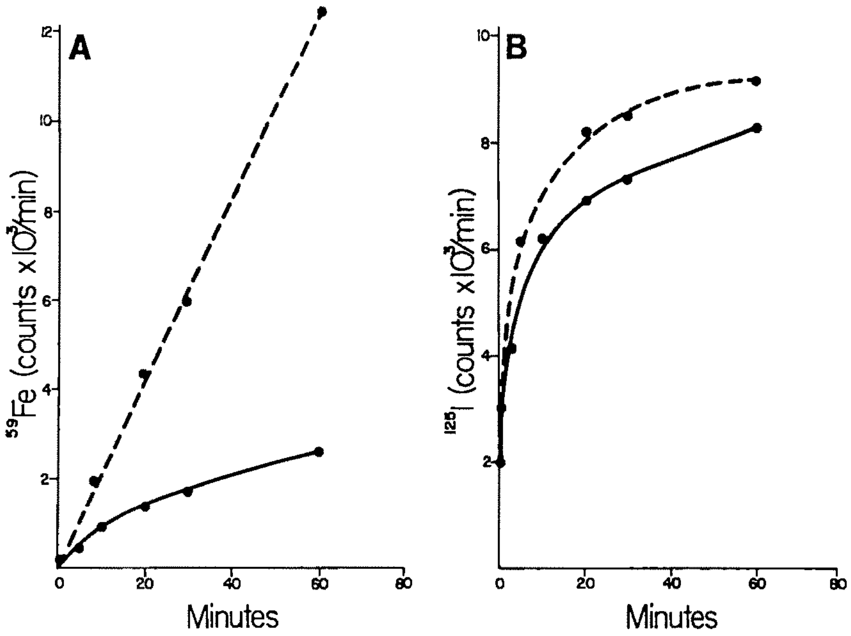

Fig 1. Uptake radioiron and ^{125}I-labeled transferrin by b/b reticulocytes (solid line) and iron-deficient reticulocytes (dashed line). Belgrade reticulocytes accumulated far less iron (a), whereas transferrin uptake appeared similar (b).

iron uptake (Fig 1). When reticulocytes from the b/b and reticulocytes from iron-deficient genotypically normal rats were incubated with iron-saturated ^{59}Fe- and ^{125}I-labeled transferrin (2 mg/mL at 37 °C), radioiron uptake was similar only during the first few minutes of incubation, and by 60 minutes, the uptake by b/b reticulocytes amounted to about one fourth that of the iron-deficient control reticulocytes. This occurred despite a similar initial ^{125}I transferrin uptake.

Iron release in the normal reticulocyte is known to involve complete removal of iron from transferrin. This aspect was examined by using a doubly-labeled rat transferrin carrying ^{55}Fe on the acid stable site and ^{59}Fe at the acid labile binding site. At incubation, the normalized uptake ratio (determined by the ^{55}Fe-^{59}Fe uptake in the reticulocyte divided by the ^{55}Fe-^{59}Fe ratio in the media) was 1.0 ± 0 01 (n = 5) for the Belgrade rat and 0.99 ± 0.01 (n = 5) for the iron-deficient control reticulocytes. There could still be a random removal of one iron from the two sites, resulting in exiting of monoferric transferrin from cells. To investigate this, ^{59}Fe-tagged diferric transferrin was used in which the protein part of transferrin was labeled with ^{125}I. After a 60-minute incubation, during which about 60% of the radioiron was removed by the iron-deficient reticulocytes and 16% by the Belgrade reticulocytes, isoelectric focusing profiles were prepared on the media (Fig 2). No accumulation of monoferric transferrin was seen in either and the amount of apotransferrin was proportional to the iron uptake by reticulocytes in the two cell mixtures.

That the abnormality involved the iron release mechanism was shown by a chase study (Fig 3). After ten minutes of preincubation of reticulocytes at 37 °C with diferric ^{55}Fe, ^{125}I rat transferrin, a 20-fold excess of nonradioactive diferric rat transferrin was added and the incubation continued for another 20 minutes. A rapid efflux of ^{125}I out of the cells was observed, essentially the same with Belgrade and iron-deficient reticulocytes. On the other hand, the behavior of ^{59}Fe was quite different (Fig 3B). No efflux of ^{59}Fe from control reticulocytes was observed, while Belgrade reticulocytes lost about 70% of the ^{59}Fe taken up during the preincubation period

This abnormal iron release became more evident when reticulocytes were exposed to EDTA or cadmium (Fig 4). Both of these inhibitors reduced iron accumulation in cells when present in millimolar concentration The effect on Belgrade reticulocytes, however, was much greater than on control reticulocytes from iron-deficient animals. No effect on iron delivery was observed when the cadmium EDTA complex (1 mmol/L Cd^{2+}, 2 mmol/L EDTA) was incubated with reticulocytes of either experimental animal (data not shown).

DISCUSSION

Belgrade rats have an autosomal recessive trait that, in its homozygous form (b/b), leads to a microcytic hypochromic anemia [2] While erythroid cells have the features of iron deficiency, including a decrease in hemoglobin content and elevated red cell protoporphyrin, some features are quite different from those expected in iron deficiency. Most conspicuous is the ample plasma iron, whereas body tissues are iron depleted. These findings suggest an iron block involving both gastrointestinal absorption and iron uptake from transferrin by body tissues. Of interest is the demonstration that the block in erythroid uptake does not appear to interfere with erythroid proliferation but does interfere with hemoglobin synthesis. Measurements by the authors on iron-deficient animals with severe anemia (mean hematocrit 15) show a total erythroid cellularity of 7 1 × 10^9/kg as compared with the mildly anemic homozygous Belgrade rats, with an erythroid cellularity of 18 × 10^9/kg. This implies that the Belgrade defect is less limited in the early phase of red cell development involving stem cell proliferation as compared with the late stages, in wich hemoglobin synthesis predominates.

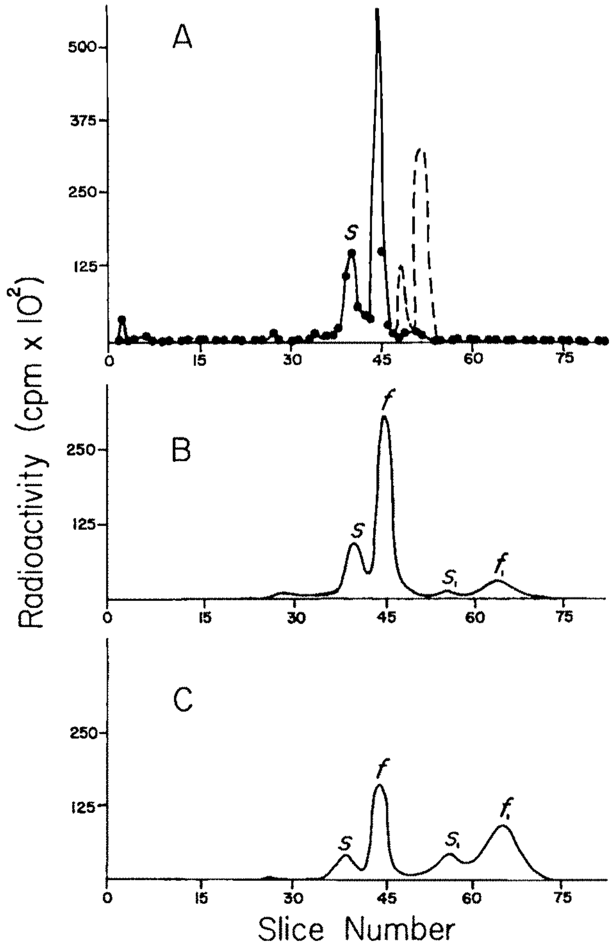

Fig 2. Chromatographic profile of ^{125}I-labeled transferrin. (A) Isoelectric focusing profile of the two diferric rat transferrins before exposure to reticulocytes. Superimposed in a dotted line is shown the locations of the two monoferric forms of rat transferrin. (B) Profile of transferrin after its incubation with b/b reticulocytes. (C) Similar profile after exposure to iron-deficient reticulocytes. Both (B) and (C) show the two peaks of slow and fast rat apotransferrin, the greater amount in (C) corresponding to the greater iron uptake by the iron-deficient reticulocytes. In neither were peaks of monoferric transferrin seen.

The extremely high plasma iron and transferrin saturation

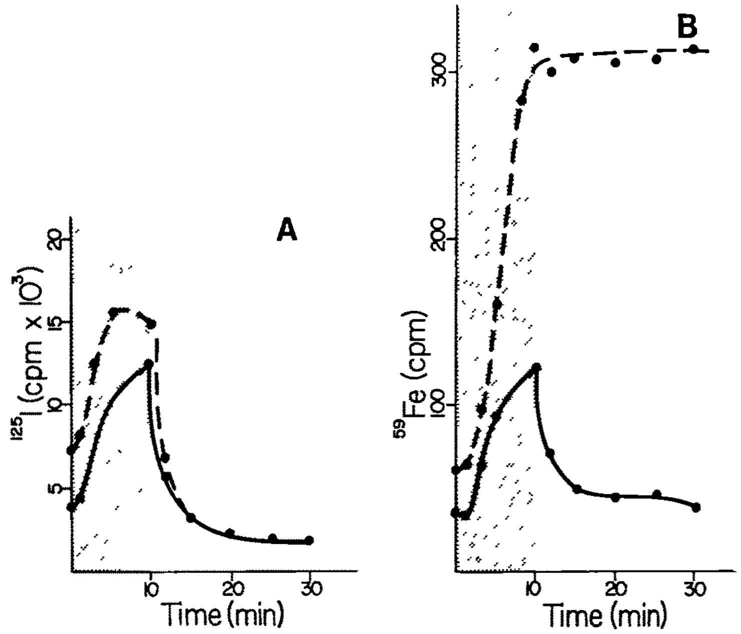

Fig 3. A chase experiment in which there was a preliminary incubation of reticulocytes with transferrin labeled both with radioiron and ^{125}I for a period of ten minutes. Thereafter, a large amount of nonradioactive diferric rat transferrin (7.9 mg containing 11 µg iron) was added to the mixture. Uptake by b/b reticulocytes is shown by solid line and that of iron-deficient reticulocytes is shown by dotted line. In the diagram on the left, the similar rate of release of transferrin from the reticulocyte mixture is seen. In the diagram on the right, a rapid release of iron is observed from b/b reticulocytes but not from normal reticulocytes.

suggest an activation of the iron procurement mechanism within the body. It has been shown that increasing the number of immature erythroid cells somehow results in an increased procurement of iron.[16] In thalassemia, the effect of erythroid proliferation is to increase absorption and total body iron, leading to parenchymal iron overload. In these animals, it would appear that total body iron cannot be increased because of an absorptive abnormality, but that the gradient between iron-donating tissues and plasma iron is altered so as to increase plasma iron supply. Because the delivery of iron to the erythron is not increased, procurement cannot be simply a replacement of iron after its removal from transferrin as previously suggested, but to some other aspect of erythroid hyperplasia.[17]

Previous studies have shown a marked shortening in red cell life span by the chromium-51 technique to seven days in b/b animals as compared with 19 days in controls.[2] These studies, however, were done with younger animals that show a severer degree of anemia. In the present study, while the number of b/b red cell precursors was about four times normal, this would represent a hemoglobin production of only 1.3 times basal, since red cells contained only about one third the hemoglobin concentration of −/? red cells. The basal turnover of plasma iron gives no indication of hemolysis and suggests, if anything, that part of the increase in erythroid cellularity in these animals might be due to a lengthened maturation time. Splenomegaly was accounted for by the marked increase in erythropoiesis within the spleen

Attention then focuses on the series of events occurring when the iron is taken up by the red cell. If there were defective unloading of iron from transferrin, this would not be seen by ferrokinetic techniques, since the shunt in and out of the red cell occurs so rapidly. The currently accepted model for transferrin-mediated delivery of iron to the mammalian erythroid cell involves a number of steps.[18] Diferric or monoferric transferrin binds to a receptor on the plasma membrane at neutral pH, and the complex is endocytosed into the cell cytoplasm. The endocytotic vesicle would be expected to contain an adenosine triphosphate-dependent proton pump, which acidifies its contents. Iron is released from transferrin in the acid environment of the vesicle and somehow transported across its membrane into the cytoplasm and ultimately to the mitochondria for incorporation into heme. The apotransferrin–receptor complex returns to the surface of the cell and fuses with the plasma membrane. On exposure to a higher pH, apotransferrin leaves the receptor and becomes available for reutilization.[19]

In the Belgrade rat reticulocyte, the initial steps involving transferrin–receptor interaction and transferrin–iron–recep-

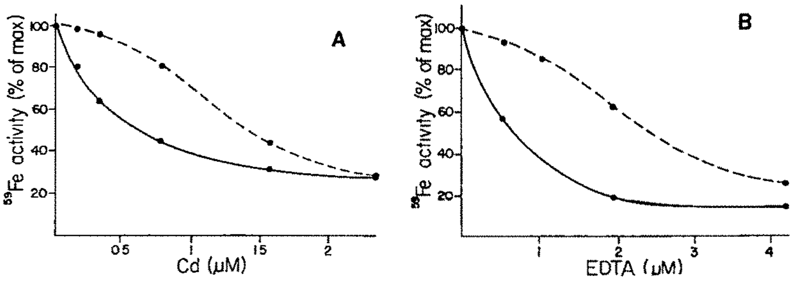

Fig 4. The effect of EDTA and cadmium on radioiron uptake by b/b (solid line) and iron-deficient (dotted line) reticulocytes. Uptake is presented as a percentage of uptake in the absence of these substances. Effects of EDTA are shown in the lower diagram and of cadmium ions in the upper diagram. With both substances, the degree of suppression of iron uptake is much greater with b/b reticulocytes.

tor internalization are intact. However, the release of iron from transferrin in the cell interior is greatly diminished. The release mechanism, although quantitatively abnormal, appears to be qualitatively normal, because when iron is removed from the cell, only apotransferrin is returned to the medium.

The release of iron from transferrin within the genotypically normal iron-deficient reticulocyte is impaired by EDTA and cadmium ions. In this regard, Belgrade reticulocytes are much more sensitive than normal reticulocytes to the effect of EDTA and cadmium ions. Because EDTA can chelate calcium and magnesium, and because cadmium, due to its chemical similarity, can compete with calcium and magnesium, it is possible that the molecular lesion in the Belgrade rat involves a calcium-dependent enzyme system concerned in the release of iron from transferrin.

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Platelet–Leukocyte Interaction: Selective Binding of Thrombin-Stimulated Platelets to Human Monocytes, Polymorphonuclear Leukocytes, and Related Cell Lines

By T.W. Jungi, M.O. Spycher, U.E. Nydegger, and S. Barandun

The association of platelets with leukocytes was investigated, using gel-filtered platelets stimulated with thrombin and then fixed with formaldehyde. Evidence is presented that stimulation of gel-filtered platelets with low concentrations of thrombin (0.01 to 0.1 U/mL) induces the expression of surface determinants interacting strongly with monocytes and polymorphonuclear leukocytes (PMNs) but only weakly with lymphocytes. Both monocyte–platelet binding and PMN–platelet binding occurred at 37 °C and more intensively at 0 °C; it was Ca^{2+}-dependent and was unaffected by the addition of sodium azide. The binding also occurred with the monocytoid cell lines HL 60 and U 937 in exponential growth and was much less two days after induction of terminal differentiation by phorbol myristate acetate (PMA). No other tested cell lines (B cells, T cells, and myeloid cells) bound thrombin-stimulated platelets. Monocyte-derived macrophages kept in culture for one week also exhibited reduced binding of thrombin-stimulated platelets. IgG and fibronectin could be ruled out as ligands that mediate binding.

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THE INTERACTION of platelets with leukocytes is poorly characterized. During isolation of mononuclear cells, platelets associate with leukocytes, particularly monocytes, but the potential physiological significance of this interaction has not been elucidated. Although earlier investigations pointed to an enhancing effect of platelets in adherence of monocytes to plastic surfaces, the requirements of this interaction were not studied in detail.[1] More recently, association of platelets with lymphocytes and monocytes hampered investigations of the surface properties[2-4] and metabolic activity[5] of these cells, and recommendations were made for obtaining platelet-free lymphocyte[2] or monocyte[3,5] preparations In addition, an increased association of platelets with monocytes and polymorphonuclear leukocytes (PMNs) (so-called satellitism) has been observed clinically, particularly in disorders of an autoimmune nature[6-8] The association of platelets with leukocytes may be complex, and various different ligands may exist, some of which are involved in a certain stage of platelet stimulation or activation only and which selectively interact with a defined leukocyte population, subpopulation, or cells of a restricted differentiation stage. In the present study, new insights are provided by study of the interaction of resting and thrombin-stimulated platelets, which are then formaldehyde-fixed, with purified lymphocyte and phagocyte populations and human cell lines. During thrombin stimulation in the presence or absence of divalent cations, platelets acquire surface properties suited for binding in a Ca^{2+}-dependent manner to monocytes and PMNs, and to established human cell lines related to both of these. Evidence against an involvement of fibronectin and immunoglobulin G (IgG) as ligands mediating the binding will be presented.

MATERIALS AND METHODS

Preparation of platelets Citrated individual blood donations collected by our blood bank were used. Buffy coats were obtained within 20 hours after collection by centrifugation at 1,200 g for five minutes at 4 °C. Three parts of buffy coats were syphoned into one part of citrate buffer (102 mmol/L of NaCl, 3 mmol/L of KCl, 30 mmol/L of Na-citrate, 4.8 mmol/L of glucose, pH 6.5) and centrifuged at room temperature for four minutes at 800 g. Platelet-rich supernatants were incubated for 30 minutes at 37 °C, pH 6.5, and gel-filtered at 37 °C on a Sepharose 2B column previously equilibrated with MES-buffer (10 mmol/L of 2-[N-Morpholino]ethanesulfonic acid [MES], 3 mmol/L of KCl, 4 8 mmol/L of glucose, 130 mmol/L of NaCl, and 2 mg/mL bovine serum albumin (BSA), pH 6.5) Gel-filtered platelets were counted on a TOA platelet counter (TOA Medical Electronics Co, Ltd, Japan) and adjusted to 2 5 × 10^9 platelets per milliliter.

For activation of platelets with bovine thrombin (61 NIH-U/mg, Hoffmann La Roche, Basel, Switzerland), the pH of platelets was adjusted to 7.4 One part of platelets was then incubated with four parts of modified Tyrode's buffer (MTB; Tyrode's buffer containing 2 mmol/L of $MgCl_2$, and 2 mg/mL BSA but no $CaCl_2$) (pH 7.4) and one part of thrombin in 0.15 mol/L of NaCl for 30 minutes at 37 °C without stirring. The incubation mixture was then added at room temperature to 18 parts of 1.3% formaldehyde in MTB, pH 6.5, without BSA, and chilled for one hour at 4 °C Fixed platelets were washed twice with MTB, pH 6 5, without BSA and finally resuspended in MTB, pH 7.4, of a concentration of 1.1 × 10^9/mL. They were stored in the presence of 0 05% Na-azide for up to two days Prolonged storage reduced the capacity of thrombin-stimulated platelets to associate with monocytes.

Preparation of monocytes. The pellets of the same buffy coats serving for platelet preparation (see above) were the starting material for preparation of monocytes. They were washed twice with citrate buffer and centrifuged at low speed (ten minutes, 200 g) to remove most of the platelets. The pellets were then resuspended in washing solution [phosphate-buffered saline (PBS) containing 13 mmol/L of EDTA, 0.1% wt/vol sodium azide, 1% wt/vol modified gelatin (Physiogel, SRK)] and subjected to centrifugation on Ficoll-Hypaque for isolation of mononuclear cells[9] These were chilled to 4 °C and washed two more times in the cold for thorough removal of platelets. Mononuclear cells (up to 50 × 10^6) were layered on top of discontinuous density gradients of Percoll with densities ranging from 1.066 to 1.060. After centrifugation (600 g for 30 minutes at 4 °C), the cell bands of a density of <1 060 and <1.062 were harvested, pooled, and subjected to further washing at

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4 °C. The final monocyte fraction consisted of 65% to 90% monocytes, as defined by staining for nonspecific esterase[10] and Turk solution; the remainder were lymphocytes. The cells were resuspended in RPMI 1640 buffered with HEPES (pH 7.2) and containing 0 5% wt/vol of BSA (RHA) Monocytes were used either in the platelet–leukocyte interaction test or in rosette formation and phagocytosis tests, which are described elsewhere [11,12] In the latter, sheep erythrocytes opsonized with IgG or treated with gelatin and fibronectin were used as targets

Isolation of PMN PMNs were obtained by collecting the top layer of the PMN-erythrocyte pellet of Ficoll-Hypaque tubes used for monocyte isolation. The layer was washed, and erythrocytes were lysed with ammonium chloride (0.83% wt/vol for 15 minutes at 37 °C). After two more washes, the PMNs were resuspended in RHA and used in the platelet interaction test; they were 98% pure, and >95% cells excluded Trypan blue.

Culture of mononuclear phagocytes Mononuclear cells were isolated from buffy coats as described, except that sterile conditions were used and washing solution was replaced by PBS containing 0.3 mmol/L of EDTA.[13] The cells were resuspended in HEPES-buffered RPMI 1640 containing 2% vt/vol pooled heat-inactivated (30 minutes at 56 °C) human group AB serum and placed in 75-cm^2 culture flasks (Falcon, Oxnard, Calif). After one hour of incubation, nonadherent cells were removed by washing with RPMI 1640, and adherent cells (≈80% to 95% monocytes) were cultured exactly as described.[14] Following an overnight culture with RPMI 1640 containing 15% heat-inactivated group AB serum, the cells were dislodged from the flask and put in bags made of teflon foil (type 100 A; Angst & Pfister, Zurich, Switzerland). The sealed bags were kept at 37 °C in an atmosphere of 5% CO_2 for one week, by which time monocytes had matured to macrophages

Culture of established cell lines. Established human cell lines of the collection of the Institute for Clinical and Experimental Cancer Research, Berne, Switzerland, were used. These included T cell lines MOLT 4 and Jurkat, B cell lines Raji and Namalva, myeloid line K562, and myelomonocytoid lines U 937 and HL 60. They were cultured in RPMI 1640 supplemented with HEPES (10 mmol/L), glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin (100 μg/mL), sodium pyruvate (1 mmol/L), and 20% fetal calf serum (FCS) for obtaining rapid growth and 10% FCS for maintenance and slow proliferation, respectively.

For induction of terminal differentiation of U 937 and HL 60 cells, 4β-phorbol 12-myristate 13-acetate (PMA) was used.[15,16] Two and a half million cells in 25-cm^2 area flasks (in 10 mL) were treated with PMA (16 mmol/L), 20 to 44 hours later, cells were harvested for use in the platelet interaction test.

Platelet–leukocyte interaction test. Fifty microliters of the platelet suspension were admixed to 50 μl of RHA and 100 μl of cell suspension adjusted to 3 to 5 × 10^6 cells/mL in 12 × 75 mm Minisorp tubes (Nunc, Roskilde, Denmark). These were incubated for 30 minutes (unless otherwise indicated) at 37 °C or 0 °C under rocking conditions Aliquots were then placed in a Neubauer chamber and immediately scored for the association of more than two platelets per cell. Another aliquot was mixed with Turk's solution and then evaluated for the platelet–cell association. In each case, 200 to 400 viable single cells were scored. In some experiments, ^{51}Cr-labeled platelets were allowed to interact with monocytes, and the incubate was separated into free platelets and leukocytes carrying bound platelets, using zonal rate centrifugation over sucrose [17] The procedure allowed determination of the mean number of platelets associating with leukocytes.

Assessment of binding of fibronectin (Fn) to gel-filtered platelets. Fn was purified by affinity chromatography on modified gelatin coupled to sepharose 4B,[18] lyophilized, and stored at 4 °C. It was pure, as determined by gel electrophoresis under reducing conditions Only one band, representing a molecular weight of 220,000, was observed Fn was labeled with ^{3}H by reductive methylation[19] and stored at −70 °C in aliquots. Specific activity was 50,000 to 70,000 cpm/μg, ^{3}H-Fn was always centrifuged for five minutes at 11,000 g and at room temperature before use.

To 50 μL of gel-filtered platelets (2.5 × 10^9/mL), 150 μL of MTB pH 7.4, 50 μL of ^{3}H-Fn in MTB, and 50 μL of thrombin in 0.15 mol/L of NaCl were added, and the tubes were incubated at 37 °C for 30 minutes without stirring. Then, 150 μL of a mixture of dinonylphthalate/dibutylphthalate (1·2 56) was underlayered, and the tubes were centrifuged at 11,000 g for 30 seconds, separating the platelets from the water phase through the phthalate mixture. Aliquots of the supernatants were counted, and the tubes were inverted. After removing traces of supernatant using absorbent paper, 150 μL of 0 5% Triton X-100 and two drops of xylol were added to the sediments prior to solubilization with a sonifier (Branson Sonic Co, Danbury, Conn). Solubilized platelets were transferred to a counting vial, and radioactivity was measured in a Packard TriCarb 2450 Liquid Scintillation Spectrometer (Downers Grove, Ill)

Interaction of Fn with gelatinized erythrocytes Erythrocytes, which were tanned and gelatinized essentially as described,[20] (E^s-T-G), were incubated with graded amounts of Fn Under the conditions used, 6% of the added Fn became bound to E^s-T-G as assessed radiometrically. E^s-T-G charged with subagglutinating Fn doses (≤10 μg/mL) were used in rosette formation assays. Agglutinating doses were used in hemagglutination inhibition tests to determine the active titer of goat anti-human Fn antibodies (purchased from Calbiochem, Behring Diagnostics, La Jolla, Calif).

RESULTS

Interaction of thrombin-stimulated, fixed platelets with monocytes. Human platelets stimulated with thrombin and then fixed with formaldehyde were assessed for their capacity to adhere to purified, platelet-poor, autologous monocytes. Although unstimulated platelets had little tendency to associate with monocytes, the platelets acquired the capacity to adhere to monocytes upon stimulation with increasing amounts of thrombin (Fig 1). This adherence was observed microscopically whether the platelet–monocyte mixtures were evaluated unstained immediately after incubation (30 minutes at 37 °C), stained with Turk's solution, or chilled and then stained. At 0.02 U/mL of thrombin, the maximal proportion of platelet-bearing monocytes was reached, but additional numbers of platelets were observed per responsive monocyte when the thrombin concentration was further increased.

The time course of the platelet–monocyte interaction at 0 °C and at 37 °C, using formaldehyde-fixed platelets previously stimulated with 0.02 U/mL of thrombin, is shown in Fig 2; a maximal proportion of platelet-bearing monocytes was observed after 10 to 15 minutes. The proportion of platelet-bearing monocytes was reproducibly higher when the interaction occurred in the cold (0 °C). Sodium azide (20 mmol/L) added to the monocyte–platelet mixtures did not influence monocyte–platelet association.

Role of plasma fibronectin (Fn) in platelet-monocyte interaction. In view of previous findings that Fn binds to stimulated platelets[21,22] and that monocytes express cell surface receptors for this protein,[20,23] a role of plasma Fn in the platelet–monocyte interaction was sought.

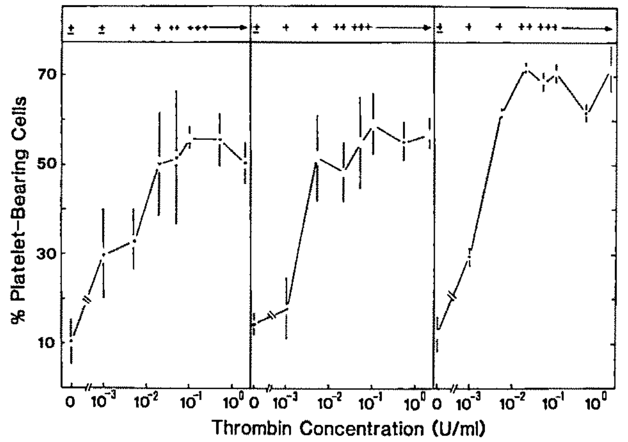

Fig 1. Thrombin-stimulated platelets, then formaldehyde-fixed, bind to monocytes. The percentage of cells bearing more than two platelets (means ± 1 SD), as assessed microscopically, after 60-minute incubation in medium RHA, is plotted as a function of the thrombin concentration used for platelet stimulation. In addition, the degree of platelet association per single monocyte was scored arbitrarily from − to + + + and is indicated in the top row panel. Left panel, unstained preparations; middle panel, cells stained with Turk's solution; right panel, tubes chilled in ice before microscopic examination.

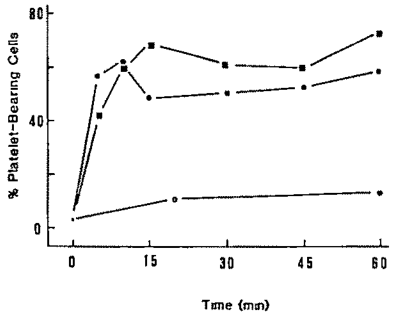

Fig 2. Kinetics of the binding of thrombin-stimulated and control platelets to monocytes in suspension. Incubation of monocytes and stimulated platelets was performed at 37 °C (●) and 0 °C (■); incubation of monocytes and control platelets was performed at 37 °C (○).

First, the amount of exogenous Fn bound to platelets stimulated by various amounts of thrombin was estimated. In agreement with previous work,[21] Fn binding reached a maximum when 0 2 U/mL thrombin was used in the presence of 2 mmol/L $MgCl_2$ while 15 mg/mL of Fn was allowed to react with 2.5 × 10^9 platelets per milliliter. Under conditions of maximal platelet–monocyte association (0.02 U/mL of thrombin), only 55 fg Fn bound per platelet. In the presence of 5 mmol/L of EDTA, binding was reduced by >90%. Using thrombin-stimulated platelets and then formaldehyde-fixed platelets rather than native platelets, saturation of ^{3}H-Fn binding was reached at the same thrombin concentration, but binding was diminished by 40% to 60%.

Second, the effect of Fn added either during thrombin stimulation of the platelets or during the interaction phase between platelets and monocytes was assessed. In both cases, exogenous Fn did not increase the proportion of platelet-bearing monocytes (Table 1, experiment 1). On the contrary, when platelet–monocyte mixtures were subjected to zonal rate centrifugation on sucrose, ^{51}Cr-labeled platelets stimulated with high thrombin concentrations in the presence of excess Fn were associated in lower numbers with monocytes than were their counterparts prepared in the absence of exogenous Fn (data not shown).

Third, goat anti-Fn antibodies were co-incubated with thrombin-stimulated platelets and monocytes. The antibodies were nonreactive with monocyte Fc receptors, as we have verified in Fc receptor-mediated phagocytosis inhibition experiments. Antibodies were shown to block the agglutination of E*-T-G mediated by Fn, yet they failed to inhibit

Table 1. Influence of Fibronectin (Fn) and Anti-Fn-Antibodies on Association of Stimulated Gel-Filtered Platelets and Monocytes

	Incubation of Platelets Before Formaldehyde Fixation		Coincubation of Formaldehyde-Fixed Platelets and Monocytes		Platelet-Bearing Cells After Incubation (%) (30 min at 37 °C)
Exp.	Thrombin	Fibronectin	Fibronectin	Anti-Fn Antiserum	Means ± 1 SD
1	—	—	—	—	9 ± 3
	—	—	300 µg/mL	—	12 ± 4
	0.1 U/mL	—	—	—	51 ± 10*
	0 1 U/mL	—	300 µg/mL	—	49 ± 11*
	—	8 µg/mL	—	—	7 ± 0
	—	8 µg/mL	300 µg/mL	—	10 ± 3
	0.1 U/mL	8 µg/mL	—	—	41 ± 6*
	0.1 U/mL	8 µg/mL	300 µg/mL	—	42 ± 3*
2	—	—	—	—	13 ± 5
	—	—	—	1:320	9 ± 2
	0.02 U/mL	—	—	—	43 ± 14*
	0.02 U/mL	—	—	1:320	44 ± 6*
	0.02 U/mL	—	—	1:640	48 ± 1*
	0 1 U/mL	—	—	—	33 ± 8*
	0 1 U/mL	—	—	1:320	42 ± 1*
	0.1 U/mL	—	—	1:640	46 ± 8*

*Significantly different from control (first line) ($P < 01$).

the association of stimulated platelets with monocytes (Table 1, experiment 2).

Role of IgG in the platelet–monocyte interaction. The possibility that IgG associating with platelets[24] interacts with monocyte Fc receptors was tested by co-incubating thrombin-stimulated platelets and monocytes with IgG at concentrations blocking Fc-receptor-mediated rosette formation and erythrophagocytosis completely. In the presence of 8 mg/mL IgG, 54% ± 7% mononuclear cells bound platelets, as compared to 43% ± 14% in the absence of IgG. Likewise, platelet–monocyte association occurred in the presence of 25% heat-inactivated serum as well (not shown).

Divalent cation requirements for platelet–monocyte association. The role of divalent cations was tested by including cation-chelating agents either during thrombin stimulation or during the platelet–monocyte interaction phase. Simultaneous exposure of platelets to EDTA and thrombin for 30 minutes prior to fixation yielded no significant reduction in monocyte adhesiveness as compared with platelets stimulated in the absence of EDTA (Table 2, experiment 1). Platelets preincubated for 30 minutes in 5 mmol/L of EDTA, then stimulated with thrombin, behaved in the same manner as did stimulated platelets not exposed to EDTA. In contrast, if either EDTA or EGTA were included during the interaction of stimulated platelets with monocytes, even fewer monocytes bound such platelets than occurred if monocytes were allowed to interact with unstimulated platelets in Ca^{2+}-sufficient and Mg^{2+}-sufficient medium (Table 2, experiment 2), suggesting that the interaction of stimulated platelets with monocytes is strictly Ca^{2+} dependent.

Binding of thrombin-stimulated platelets to various cell types derived from human blood Even if platelets had little tendency to bind to lymphocytes contaminating the monocyte preparation, it was possible that certain lymphocyte subpopulations absent from the tested density fractions also had the capacity to bind stimulated platelets. Therefore, lymphocyte-enriched cell fractions were also washed and tested for their capacity to bind stimulated platelets. Although in all fractions (except Percoll density-gradient fraction II), most monocytes bound stimulated platelets, only a small subpopulation of lymphocytes (5% to 10%) did so (Fig 3). Such lymphocyte–platelet interaction, however, was largely resistant to a subsequent treatment with EDTA.

In another set of experiments, the capacity of purified PMNs to bind stimulated platelets was tested. PMNs appeared to be capable of binding stimulated platelets, but higher thrombin concentrations (0.1 U/mL) were required for rendering platelets optimally adhesive to PMNs (Fig 4). In addition, the number of platelets per PMN was consistently smaller than the number binding to monocytes. The platelet–PMN association was also observed at 0 °C, but not in the presence of EDTA (Table 3).

Binding of thrombin-stimulated platelets to cultured cells. Freshly isolated monocytes were further matured to macrophages by means of teflon bag cultures.[14] Mature macrophages had little tendency to bind stimulated platelets, despite their increased surface area (Table 4). The same macrophages eagerly ingested erythrocytes sensitized with IgG or with IgM and complement (not shown). Established cell lines of various origin were also tested for their capacity to bind thrombin-stimulated platelets. Table 4 shows that the monocyte-like cell lines (U 937 and HL 60) bound thrombin-stimulated platelets. The tendency to bind platelets was also Ca^{2+} dependent and restricted to thrombin-stimulated platelets. Preferential binding was observed with cells in exponential growth; when terminal differentiation of U 937 and HL

Table 2. Effect of Divalent Cations on Association of Thrombin-Stimulated Platelets and Monocytes

Exp	Pretreatment of Platelets		Addition to Platelet–Monocyte Incubate at 37 °C	Platelet-Bearing Cells After 30 min (%) (Mean ± 1 SD)
	First 30 min at 37 °C	Second 30 min at 37 °C		
1	—	—	—	15 ± 4
	—	EDTA	—	16 ± 2
	—	Thrombin (0.02 U/mL)	—	43 ± 14†
	—	EDTA + Thrombin (0 02 U/mL)	—	47 ± 6†
	EDTA‡	EDTA + Thrombin (0 02 U/mL)	—	47 ± 4†
	—	Thrombin (0.1 U/mL)	—	33 ± 8†
	—	EDTA + Thrombin (0.1 U/mL)	—	42 ± 12†
	EDTA	EDTA + Thrombin (0 1 U/mL)	—	53 ± 4†
2	—	—	—	9 ± 3
	—	—	EDTA*	6 ± 2
	—	—	EGTA*	13 ± 3
	—	Thrombin (0.1 U/mL)	—	51 ± 10†
	—	Thrombin (0.1 U/mL)	EDTA	11 ± 5
	—	Thrombin (0.1 U/mL)	EGTA	6 ± 2
3	—	Thrombin (0.02 U/mL)	—	49 ± 1
	—	Thrombin (0.02 U/mL)	EGTA*	9 ± 2‡
	—	Thrombin (0 1 U/mL)	—	52 ± 16
	—	Thrombin (0 1 U/mL)	EGTA*	2 ± 1‡

*5 mmol/L (experiments 1 and 3) or 4 mmol/L (experiment 2).

†Significantly higher than negative control (first line of this experiment) ($P < .01$).

‡Significantly lower than positive control (first line of this experiment) ($P < .01$)

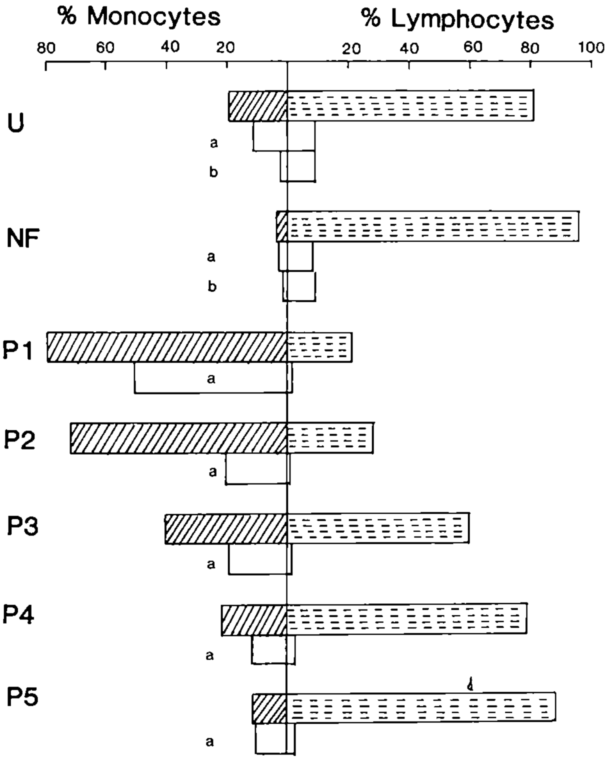

Fig 3. Binding of stimulated platelets by monocytes and lymphocytes in unseparated mononuclear cells (U), in nonadherent mononuclear cells (NF), and in different density fractions recovered from a discontinuous Percoll gradient The hatched and stippled bars show the proportion of monocytes and lymphocytes, respectively, in each fraction; the open bars show the proportion of monocytes and lymphocytes, respectively, within the whole fraction binding more than two platelets in the absence (a) or presence (b) of 5 mmol/L of EDTA.

60 was induced by PMA, the capacity of these cells to bind stimulated platelets gradually waned. No other nonmonocytoid cell line was capable of binding thrombin-stimulated platelets. Thus, the interaction of stimulated platelets with cellular surfaces is restricted to surfaces of professional phagocytes in certain defined stages of differentiation and to that of established cell lines closely related to phagocytes.

DISCUSSION

The interactions between platelets and phagocytes are ill-defined. Although platelets are the first cellular elements to get involved in areas in which vascular basement membrane has been exposed, phagocytes soon follow.[1] Phagocytes essentially determine the cellular and molecular composition of inflamed tissue, and platelets too are found in areas of inflammation such as the synovial tissue of rheumatoid arthritis patients.[25] Phagocytes generate mediators affecting platelets,[26] and both phagocytes and platelets are capable of producing and responding to a variety of substances of the arachidonic acid metabolism.[5,27,28] Furthermore, the surface receptor spectra of phagocytes and platelets have much in common with each other.[20-24,29,30] In the laboratory, platelets often contaminate preparations of phagocytes, particularly monocytes, and special procedures are necessary to prevent association of platelets with monocytes.[3,5,31]

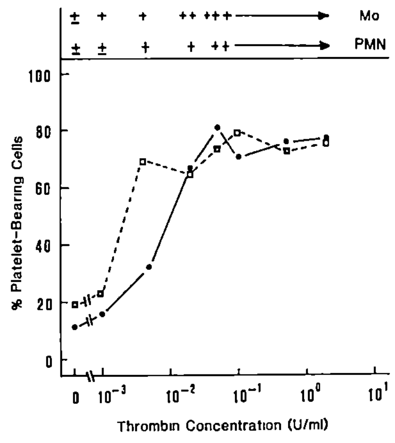

Fig 4. Binding of stimulated platelets, then formaldehyde-fixed, to PMNs as a function of the thrombin concentration used for stimulation. Purified PMN were incubated for 30 minutes at 37 °C and then assessed after being stained with Turk's solution (●). For comparison, the proportion of platelet-bearing monocytes in a parallel experiment with the same platelets is shown (□). The degree of platelet-association per single PMN or monocyte, respectively, is expressed in arbitrary scores from — to + + + and is indicated in the top panel.

In view of these considerations, it was not unexpected when we found that, in vitro, platelets associated with monocytes and PMNs in a specific and reproducible fashion. The finding is based on studies with gel-filtered platelets that were stimulated with thrombin under conditions that did not lead to aggregation and that were then fixed with formaldehyde. This fixative agent, which cross-links certain surface determinants, leaves various platelet properties unaffected, including antigenic properties,[32] the capacity to bind myxoviruses,[33] reactivity in lectin-induced aggregation tests,[34] and involvement in aggregation when precoated with fibrinogen and co-incubated with native platelets and thrombin.[35] Yet formaldehyde-fixed platelets fail to aggregate upon stimulation with thrombin[34] and thus are suitable for studying their interaction as single particles with leukocytes.

Platelets acquired adhesiveness to monocytes when stimu-

Table 3. Binding of Stimulated Gel-Filtered Platelets to PMNs occurs at 37 °C and at 0 °C, but Not in the Presence of EDTA

Incubation of Platelets Prior to Fixation	Conditions During Platelet- PMN Interaction		Platelet-Bearing Cells (%)	
	EDTA	Temp.	Unstained	Turk-stained
Buffer	—	0 °C	5 ± 2*	6 ± 3
Thrombin 0.1 U/mL	—	0 °C	54 ± 18†	41 ± 7‡
Thrombin 0.1 U/mL	5 mmol/L	0 °C	8 ± 0	17 ± 7
Buffer	—	37 °C	14 ± 4	14 ± 10
Thrombin 0.1 U/mL	—	37 °C	61 ± 4†	40 ± 14†
Thrombin 0.1 U/mL	5 mmol/L	37 °C	5 ± 4	15 ± 1

PMNs, polymorphonuclear leukocytes

*Means ± 1 SD of triplicate evaluations of the same experiment.

†Significantly different ($P < .05$) from control (lines 1 and 4, respectively)

Table 4. Interaction of Stimulated Gel-Filtered Platelets and Cultured Cells

Treatment of Platelets Prior to Fixation	Tyrode	Thrombin (0.02 U/mL)		Thrombin (0.1 U/mL)	
	37 °C	37 °C	0 °C	37 °C	0 °C
	Platelet-Bearing Cells (%) (Means ± SD)				
Tested Cells					
Monocytes	6 ± 3	71 ± 5*		69 ± 0	
Macrophages (7 d, 1 °C culture)		7 ± 5	9 0	7 ± 1	18 ± 6
HL 60	4 ± 3	84 ± 4		70 ± 6	88 ± 7
HL 60 PMA-ind. (20 h)		63 ± 4			
HL 60 PMA-ind. (44 h)		24 ± 3		23 ± 6	5 ± 3
HL 60 + EGTA†		2 ± 3			
U 937	10 ± 1	84 ± 3		58 ± 11	93 ± 6
U 937 PMA-ind. (20 h)		70 ± 3			
U 037 PMA-ind. (44 h)		20 ± 4		22 ± 1	7 ± 1
U 937 + EGTA†		3 ± 1			
K 562		3 ± 2	3 ± 1	5 ± 2	7 ± 2
MOLT 4		4 ± 2	3 ± 1	1 ± 0	5 ± 0
Jurkat		5 ± 1	6 ± 2	5 ± 2	4 ± 2
Raji		6 ± 4	5 ± 1	2 ± 1	3 ± 1
Namalva		2 ± 2	8 ± 1	2 ± 0	2 ± 1

PMA-ind., 4β-phorbol 12-myristate 13-acetate–induced (induction time in brackets).

*Means ± 1 SD of triplicate evaluations of the same experiment.

†EGTA (5 mmol/L) present during coincubation of cells with gel-filtered platelets.

lated with thrombin in a dose-dependent manner. The lowest dose of thrombin which induced a significant adhesiveness to monocytes was 0.001 U/mL; <0.02 U/mL yielded an optimal response. For adherence to neutrophils, thrombin concentrations ten times higher were required to achieve the same binding properties. Thrombin-stimulated platelets directly bound to phagocytes rather than to contaminating native platelets; the latter had been thoroughly removed during phagocyte purification. Unstimulated, fixed platelets had little tendency to associate with phagocytes; it is our assumption that the few platelets in an unstimulated population which nevertheless adhered represented a subpopulation that had undergone spontaneous stimulation, most probably during centrifugation. This assumption is supported by the finding of Perussia et al[3] that the association of monocytes with platelets in Ficoll-Hypaque–purified mononuclear cell preparations can be prevented by pretreating the blood with aspirin and EDTA, thus limiting spontaneous platelet stimulation.

Which are the surface determinants on stimulated platelets that mediate the observed binding? Although further studies must solve this question, we can at least rule out some of the possibilities. For example, fibronectin does not appear to be involved, although Fn is released during thrombin stimulation and is bound by the platelet surface[21,22,36] and although phagocytes do have receptors for Fn.[20,23] The arguments against Fn involvement in our model are several-fold. Because platelet–monocyte association occurs without the addition of Fn, Fn would have to be surface-bound either on platelets or on monocytes. That it would be bound to monocytes is highly improbable since our conditions of monocyte washing (in the presence of EDTA) would appear to prevent this, and the time interval in the interaction test would be too short to allow Fn synthesis by monocytes. Moreover, gelatinized erythrocytes were not rosetted by monocytes (data not shown), although Fn may serve as a bridging molecule between gelatin and monocytes (ref. 20 and this study), and although the same erythrocytes avidly bound radiolabeled Fn. Thus, Fn would have to be surface-exposed by platelets during thrombin stimulation. However, little Fn binding and insignificant serotonin release were observed when platelets were stimulated with 0.02 U/mL of thrombin although these platelets were optimally adhesive to monocytes. Moreover, the addition of free Fn to platelet–monocyte mixtures did not inhibit the binding as would be expected if free ligand were to compete with surface-bound Fn. Regardless whether a putative Fn ligand were of platelet or monocyte origin, its ligand role would be affected by co-incubation with anti-Fn antibodies but not with the selective Ca^{2+} chelator EGTA.[20] However, platelet–monocyte interaction was blocked completely by 5 mmol/L of EGTA (Table 2), proving its strict Ca^{2+} dependency during the platelet–monocyte interaction phase; in contrast, it was unaffected by the addition of Fn-antibodies at concentrations effectively blocking the agglutination of gelatinized erythrocytes in the presence of Fn. Our findings do not rule out the possibility that Fn may participate in the platelet–monocyte interaction under other experimental conditions.

Another adhesive protein involved in platelet function is fibrinogen (Fg).[37] Formaldehyde-fixed platelets were shown to interact with fresh, stimulated platelets by virtue of their Fg receptor, recognizing Fg on their native counterparts.[37] Moreover, mononuclear phagocytes were reported to bind fibrin and/or fibrinogen specifically,[38-40] and it was suggested recently that human monocytes express the GP IIb/IIIa complex, the platelet Fg receptor.[41,42] However, this hypothesis is still controversial,[4] and our preliminary results did not favor an involvement of Fg in platelet–monocyte interaction. In these experiments, ADP-stimulated, fixed platelets that are known to express Fg receptors[37] failed to adhere to monocytes regardless of the amount of exogenous Fg added during stimulation (data not shown).

A further potential mediator for platelet–monocyte interaction is IgG, since monocytes bear IgG-Fc receptors and platelets have been reported to carry significant amounts of platelet-associated IgG. In particular, a cohort of aged platelets is said to carry increased amounts of IgG, probably natural antibodies against structures exposed on old platelets.[43] However, several lines of evidence militate against an involvement of IgG in the platelet–monocyte interaction: First, EDTA abolishes the adherence of platelets to monocytes (Table 2) and PMNs (Table 3) but not the binding of IgG coated targets, including platelets[17] to monocytes. Second, the described adherence phenomenon also occurs in the presence of 25% normogammaglobulinemic heat-inactivated human serum or 8 mg/mL IgG. However, both completely block the monocyte rosette formation with IgG-coated erythrocytes (T.W. Jungi, unpublished observations, 1983). Third, the binding of IgG-coated particles provides an obligatory signal for internalization of the bound particle, but neither we nor others[5] observed internalization of monocyte-bound platelets. Finally, it is difficult to comprehend how thrombin stimulation would alter the reactivity of platelets in such a dramatic way if IgG were the ligand mediating the binding. This evidence against involvement of IgG in the described platelet–monocyte interaction does not exclude the possibility that certain forms of platelet satellitism observed clinically are caused by platelet-specific antibodies or platelet-associated immune complexes.[44]

Thrombin-stimulated platelets seem to adhere to monocytes and PMNs without engaging their metabolism, because the adherence reaction occurs rapidly regardless of the temperature or of the presence of sodium azide. However, it is selective, insofar as it was seen with monocytes and PMN exclusively. Only a small lymphocyte subpopulation (which was not further characterized) bound stimulated thrombocytes; this binding was not Ca^{2+} dependent. Monocytes that had matured to macrophages in vitro exhibited a diminished capacity to bind stimulated platelets despite an increased activity in ingesting IgG-sensitized target cells. On the other hand, cell lines representing undifferentiated precursors of monocytes and/or neutrophils avidly bound stimulated platelets; this binding was also Ca^{2+} dependent and occurred at 0 °C as well as at 37 °C. Because cultured macrophages are highly adherent but the above cell lines are not, the capacity to bind stimulated platelets is not an expression of the adherence properties of phagocytes. Because all other cell lines tested, representing precursors of the T cell, B cell, and erythrocyte lineage, were incapable of binding stimulated gel-filtered platelets, it appears that the capability of binding thrombin-stimulated platelets is a property unique to monocytes, PMNs, and cell lines related to them. The presence of a monocyte subpopulation failing to bind platelets (Figs 1 and 3) and the reduced efficiency of cultured macrophages and PMA-treated myelomonocytoid cell lines in the platelet-binding test suggest that the capacity of phagocytes to interact with thrombin-stimulated platelets is further restricted to certain stages of differentiation.

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Amplification of Genes Encoding Human Myeloid Membrane Antigens After DNA-Mediated Gene Transfer

By A. Thomas Look, Stephen C. Peiper, Edwin C. Douglass, Jeffrey M. Trent, and Charles J. Sherr

Spontaneous amplification of genes encoding two different human myeloid surface antigens was observed after DNA-mediated gene transfer of cellular DNA from the human myeloid cell line HL-60 into NIH-3T3 mouse fibroblasts. Transformed recipient cells with highly amplified expression of either of two donor membrane polypeptides, gp150 or p67, were isolated with a fluorescence-activated cell sorter (FACS), using monoclonal antibodies specific for human myeloid cells. Immunoprecipitation of enzymatically radioiodinated polypeptides from the surface of transformed NIH-3T3 cells confirmed that expression of these proteins was amplified tenfold to 20-fold in comparison to their expression on human myeloid cell lines. The cellular DNA of cloned secondary and tertiary transformants expressing high levels of gp150 and p67 contained amplified sets of DNA restriction fragments that hybridized with human repetitive DNA sequences. Cytogenetic analysis of subclones overexpressing gp150 revealed extrachromosomal double minutes (DMs), whose presence correlated with the unstable expression of the membrane polypeptide. Human sequences in gp150-positive clones did not localize to chromosomes, consistent with their association with extrachromosomal DMs. By contrast, p67-positive subclones stably expressed the antigen, and in situ hybridization to metaphase spreads demonstrated that amplified human DNA sequences were integrated into a specific marker chromosome. Cytogenetic analysis of the parental NIH-3T3 subclone used in these studies disclosed DMs in a low percentage of metaphases, suggesting that the recipient cells have a propensity for amplifying donor DNA.

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GENE amplification is an important mechanism for the development of drug resistance in cultured cells exposed to antimetabolites.[1-4] In addition, certain tumor cell lines have been found to contain amplified cellular oncogenes, including N-*myc*,[5-7] c-*myc*,[8,9] and c-*abl*,[10] which presumably confer a proliferative advantage to cells both in vitro and in vivo. When gene amplification is manifested by aberrant banding regions (ABRs), expression of the amplified DNA sequences tends to be stable.[11] If double minute chromatin bodies (DMs) are involved, there is unequal segregation of the amplified genes under nonselective conditions, resulting in an unstable phenotype.[4,12]

Amplification of genes can also occur spontaneously. For example, methotrexate-resistant cells with a 50-fold increase in copies of the dihydrofolate reductase gene were isolated with a fluorescence-activated cell sorter (FACS) after staining with a fluoresceinated conjugate of methotrexate.[13] Similarly, spontaneous amplification of the gene encoding the human T cell membrane antigen Leu-2 (or T8) was observed after DNA-mediated gene transfer of human DNA sequences into mouse cells followed by flow cytometric selection of Leu-2-positive recipients with a fluorescein-conjugated antibody.[14,15] In the latter case, high but unstable levels of expression of the Leu-2 antigen in recipient cells were associated with the presence of DMs, implying that the transferred gene was amplified extrachromosomally

We recently used DNA-mediated gene transfer and cell-sorting procedures to isolate mouse cell clones that had acquired human DNA sequences coding for a myeloid differentiation antigen, gp150.[16] Mouse NIH-3T3 cells were co-transfected with high-mol-wt DNA from the human HL-60 promyelocytic cell line together with a molecularly cloned feline sarcoma virus (FeSV) containing the v-*fms* oncogene. The v-*fms* transforming gene, which served as the dominant selectable marker, permitted cells that had acquired exogenous DNA to overgrow nontransformed fibroblasts. Because most transformed cells incorporated ~1,500 kilobases (kb) of human DNA, or ~0.1% of the human genome,[17] we were able to select antigen-positive recipient cells by automated cell-sorting procedures performed with four monoclonal antibodies directed to different gp150 epitopes. Sequential rounds of DNA-mediated gene transfer and cell sorting were used to isolate a family of secondary and tertiary mouse cell transformants, each of which contained a limited but characteristic set of human DNA restriction fragments presumed to represent the gene coding for gp150.

In the course of these experiments, we identified recipient mouse cell clones that expressed significantly higher levels of gp150 than did the human HL-60 donor cells. Experiments performed with another myeloid antigen, p67, yielded similar results. We now show that the relative overexpression of both gp150 and p67 in recipient mouse cells is associated with specifically amplified human gene sequences The amplified sequences appeared to be associated with DMs in recipient cells that unstably overexpressed gp150 and were integrated at a specific chromosomal locus in cells demonstrating stable amplification of p67.

MATERIALS AND METHODS

Cell lines. NIH-3T3[18] [subclone 7 derived by Dr Douglas Lowy[19]] and L (*tk*−) cells[20] were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10%

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fetal calf serum (FCS), penicillin (100 μg/mL), and streptomycin (100 μg/mL). The human myeloid cell lines HL-60[21] and KG-1a[22] were maintained in RPMI 1640 medium containing 15% FCS and antibiotics. Transformed L cells were grown in selective media containing hypoxanthine (100 μmol/L), aminopterin (0.4 μmol/L), and thymidine (16 μmol/L) (HAT). Transformed NIH-3T3 cells were cultured in medium containing 5% FCS

Transfection of high-mol-wt DNA. DNA from the cloned McDonough strain of FeSV containing the v-*fms* oncogene[23] was co-transfected with 5 μg of sheared cellular DNA into NIH-3T3 cells, using the calcium phosphate technique.[19,24] After 18 hours of incubation with DNA precipitates, the cells were trypsinized and plated into 75-cm^2 T-flasks with medium containing 5% FCS. Foci of transformed cells were enumerated 12 days later and were then allowed to overgrow the monolayer of untransformed NIH-3T3 recipients. The efficiency of transformation in this system is ~2,000 foci per microgram of FeSV DNA[23,25] L (*tk*−) cells were co-transfected with the herpes simplex *tk* gene and 5 μg of sheared cellular DNA. After 18 hours of incubation, the cells were trypsinized and seeded into 75-cm^2 T-flasks. HAT medium was added 24 hours later and was changed every three or four days. Transfected NIH-3T3 or L cells from 7 to 20 confluent flasks were trypsinized and pooled for selection by FACS.

Monoclonal antibodies and flow cytometry. Transformants were sorted by flow cytometry after staining with either of two monoclonal antibodies (MY7 or MY9; Coulter Immunology, Hialeah, Fla) that react with epitopes of surface molecules expressed by human myeloid cells.[26-28] Transformants isolated with the two antibodies were tested by flow cytometry for binding of the monoclonal antibodies DU-HL60-4,[29] obtained from Dr Richard S Metzgar of Duke University (Durham, NC); MCS.2[30] obtained from Dr Jun Minowada of Loyola University (Chicago, Ill), SJ-D1,[31] obtained from Dr Joseph Mirro of St Jude Children's Research Hospital (Memphis, Tenn); and L4F3 and L1B2[32] obtained from Dr Irwin D Bernstein of the Fred Hutchinson Cancer Research Center (Seattle, Wash) NIH-3T3 transformants were also tested for binding of the monoclonal antibody SM2.6.3, which recognizes an epitope of the cell surface glycoprotein encoded by the v-*fms* oncogene.[25,33] Indirect immunofluorescence labeling, flow cytometric analysis, and cell sorting were performed as previously described.[25]

Detection of human repetitive sequences in transfected mouse cell DNA. Cellular DNAs were digested with various restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md), separated by electrophoresis in 0.8% agarose gels, and transferred to nitrocellulose [34] Human DNA containing highly repetitive sequences was prepared, labeled to a specific activity of 1×10^8 dpm/μg by nick translation, and used in blot hybridization under stringent annealing conditions.[16]

Radioiodination of cell surface proteins. Live HL-60, KG-1a, or transformed NIH-3T3 cells were iodinated with lactoperoxidase, and detergent lysates were immunoprecipitated and separated by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS).[16] The positions of radiolabeled proteins were determined by autoradiography of the dried slab gels by using protein standards of known mol wt.

Cytogenetic analysis and in situ hybridization Chromosomes were harvested from colcemid-treated NIH-3T3 transformants, and air-dried slides were prepared.[35] The autoradiographic images of 50 mitotic cells from the gp150 and p67 positive clones were examined following in situ hybridization with ^{3}H-labeled human repetitive DNA by methods previously described.[36] Prior to in situ hybridization, the cells were stained with quinacrine (Q-banding) and were photographed for positive reference Cells were then hybridized for 16 5 hours at a final DNA concentration of 0 5 μg/ml, followed by exposure for 14 days and subsequent autoradiographic development

RESULTS

Isolation of mouse cell transformants overexpressing human myeloid cell surface antigens. DNA from the human promyelocytic cell line, HL-60, was co-transfected into mouse NIH-3T3 cells with the cloned v-*fms* gene, and recipient cells were isolated by flow cytometry, using monoclonal antibodies to myeloid-specific antigens. Serial rounds of transfection and sorting[16] were used to obtain two classes of tertiary transformants that reacted with the gp150-specific monoclonal antibody, MY7. The two classes of MY7-positive cells included a major population with fluorescence intensity similar to that seen with donor HL-60 cells and a minor population with markedly increased fluorescence. When agar subclones derived from these cell populations were retested for MY7 binding, most showed a homogeneous pattern of MY7 antibody fluorescence, similar to that observed in primary and secondary transformants (results with subclone SJ-150-A in Fig 1, panel A). By contrast, a small proportion of the subclones showed increased MY7 binding with a heterodisperse pattern of fluorescence intensity. The brightest cells from one of these subclones (SJ-150-B) were sorted three more times, and the resulting population was enriched for highly fluorescent cells (Fig 1, panel A). Amplified gp150 expression by subclone SJ-150-B was also observed with three other gp150-specific antibodies, DU-HL60-4, MCS.2, and SJ-D1 (data not shown).

In parallel experiments, we used the monoclonal antibody MY9[28] to test recipient mouse cells for the expression of another myeloid differentiation antigen, a polypeptide of 67 kd that is also expressed on the surface of HL-60 cells. This antigen is provisionally designated "p67" because the nature of its posttranslational modification, if any, is presently unclear. Pooled L cell transformants co-transfected with HL-60 DNA and the cloned herpes simplex thymidine kinase gene were selected in HAT medium, and MY9-positive populations were isolated after cell sorting. DNA from a primary transformant selected for reactivity with MY9 was then co-transfected with the v-*fms* gene into NIH-3T3 cells, and pooled secondary transformants were sorted with the same monoclonal antibody. After five rounds of sorting, the selected cells were uniformly positive for MY9 binding. Retesting of subclones derived from agar colonies demonstrated a threefold increase in fluorescence as compared to the negative autofluorescence profile obtained with the control myeloma protein, as shown for subclone SJ-67-A in Fig 1, panel C.

Although cells with amplified expression of MY9 were not evident in the pooled transformants after five rounds of sorting, the brightest 2% of cells were repeatedly sorted to isolate occasional cells with amplified p67 expression. After six more rounds of sorting, subclones showed uniformly exaggerated levels of MY9 binding and stably expressed high levels of antigen after several months in continuous culture. The fluorescence histogram for one of the latter subclones, SJ-67-B, showed ~40-fold more MY9-specific fluorescence than did control cells stained with myeloma

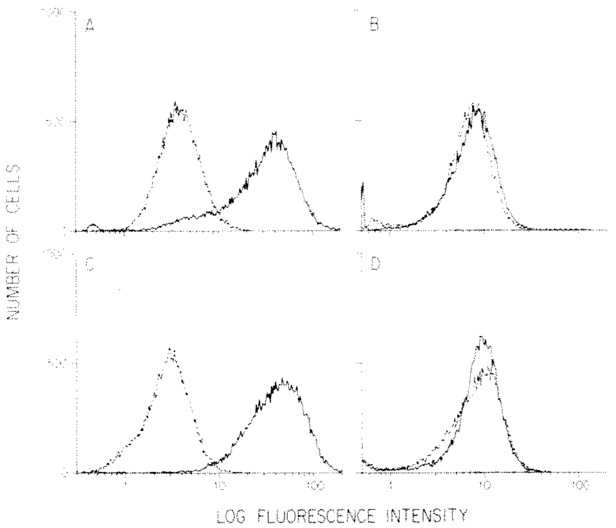

Fig 1. Cell surface expression of human myeloid membrane antigens and the v-*fms*-coded glycoprotein detected by flow cytometry. In each panel, the fluorescence profiles recorded with antibodies specific for epitopes of human myeloid antigens or v-*fms*-coded molecules are compared with the control fluorescence profile obtained with an isotype-matched mouse or rat myeloma protein (..........). Fluorescence profiles obtained with the gp150-specific monoclonal antibody MY7 with transformed NIH-3T3 subclones SJ-150-A (--------) and SJ-150-B (————) are shown in panel A. Analogous results using the p67-specific monoclonal antibody MY9 and subclone SJ-67-A (---------) and SJ-67-B (————) are shown in panel C. Results using monoclonal antibody SM 2.6.3 to a v-*fms*-coded epitope are shown in panels B (SJ-150 clones) and D (SJ-67 clones). Symbols designating the cell lines are the same as those in panels A and C, respectively.

protein (panel C). This subclone also exhibited high levels of binding with the monoclonal antibodies L1B2 and L4F3, whose patterns of reactivity with human myeloid cells are similar to those of MY9. Thus, MY9, L1B2, and L4F3 appear to bind to epitopes encoded by the same human gene.

Because the v-*fms* gene also encodes a transforming glycoprotein that can be detected on the cell surface by flow cytometry,[25,33] we tested each of the four subclones shown in Fig 1 for expression of the v-*fms* gene product. Flow cytometric analysis of surface immunofluorescence using the monoclonal antibody SM2.6.3, specific for a v-*fms*-coded epitope, revealed similar intensities of binding with each of the subclones (panels B and D), in contrast to the marked differences observed for the binding of the antibodies MY7 or MY9. Thus, independently derived subclones exhibited nearly identical levels of the transforming gene product on the cell surface, irrespective of amplified expression of human myeloid antigens.

Although certain NIH-3T3 cell clones showed extremely high levels of binding with monoclonal antibodies MY7 (to gp150) and MY9 (to p67), we were unable to select L cell transformants that "overexpressed" these antigens. After repeated selection of the brightest L cell recipients in multiple rounds of sorting, the fluorescence profiles remained similar to those found with the NIH-3T3 subclones SJ-150-A and SJ-67-A and with donor HL-60 cells. The results obtained with the different recipient cells and dominant selectable markers may reflect a greater propensity of NIH-3T3 cells to overexpress these antigens. From the similar efficiency of transformation by the two different dominant selectable markers,[16] it seems unlikely that these differences arose from screening a greater number of transfection events in the v-*fms*/NIH-3T3 cell system *v* the tk/L cell system.

Immunoprecipitation of the overexpressed surface antigens. Two glycoproteins of 130 kd (gp130) and 150 kd (gp150) can be specifically immunoprecipitated with the antibodies MY7 and DU-HL60-4 from metabolically labeled lysates of both donor HL-60 cells and recipient mouse cell transformants. Kinetic and biochemical analyses established that gp130 is an intracellular precursor that differs in its carbohydrate moiety from gp150, the mature form of the glycoprotein detected on the cell surface.[16] To confirm the flow cytometric evidence for amplified expression of gp150, we radioiodinated live cells with lactoperoxidase and immunoprecipitated detergent lysates with the antibody DU-HL60-4. Figure 2A shows that gp150 molecules were specifically precipitated from plasma membranes of HL-60 cells and from the tertiary transformed NIH-3T3 subclones, SJ-150-A and SJ-150-B. As expected from the flow cytometric results, approximately tenfold more gp150 molecules were precipitated from SJ-150-B cells, as compared with results obtained with an equivalent number of SJ-150-A cells.

The molecular species recognized by the monoclonal antibody MY9 was studied in the human myeloid cell line KG-1a and the MY9-positive secondary transformants SJ-67-A and SJ-67-B. Figure 2B shows that the p67 polypeptide was barely detectable in radioiodinated membranes from either KG-1a cells or SJ-67-A cells. Similar low levels of expression occurred when HL-60 cells were used (data not shown). By contrast, subclone SJ-67-B showing stable amplification of MY9 expression (Fig 1) yielded at least 20-fold more p67 molecules than did equivalent numbers of KG-1a or SJ-67-A cells. In this case, secondary transformants expressing high levels of p67 should prove to be of particular value in the biochemical analysis of the p67 polypeptide.

Amplification of human repetitive DNA sequences. The cellular DNA of secondary mouse cell transformants selected for expression of gp150 possesses a characteristic set of human DNA restriction fragments that are recognized by a probe specific for human repetitive DNA.[16] Because the DNA of independently derived secondary transformants yielded the same human DNA restriction fragments, the human DNA sequences shared by these clones were presumed to encode gp150. To determine if amplified expression of gp150 by tertiary transformants resulted from amplification of the transfected human gene, we performed Southern blot analysis of cellular DNA from subclones SJ-150-A and SJ-150-B (Fig 3). After digestion with the restriction endonuclease *Hind* III, six human DNA restriction fragments were detected in both subclones (lanes 1 and 2). The intensity of hybridization of a human repetitive sequence probe with DNA from subclone SJ-150-B was approximately tenfold greater than that observed with an identical amount of DNA from subclone SJ-150-A, suggesting that

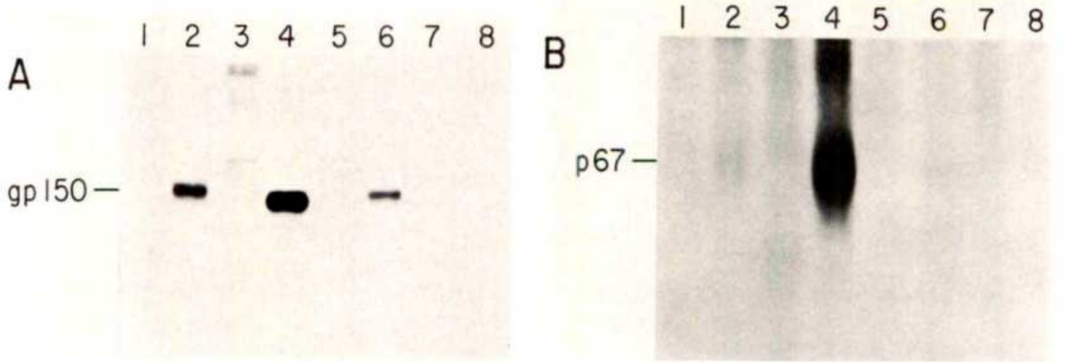

Fig 2. Expression of gp150 and p67 on the plasma membrane of human myeloid cell lines and NIH-3T3 transformants. Viable cells were enzymatically radioiodinated, and detergent lysates were immunoprecipitated. Labeled proteins in immune complexes were denatured and analyzed in polyacrylamide gels containing sodium dodecyl sulfate. Each lane represents immunoprecipitates prepared from 3×10^6 cells. In panel A, lysates of radioiodinated HL-60 cells (lanes 1 and 2), subclone SJ-150-B (lanes 3 and 4), subclone SJ-150-A (lanes 5 and 6), and control v-*fms* transformed NIH-3T3 cells (lanes 7 and 8) were precipitated with control myeloma protein (lanes 1, 3, 5, and 7) or the monoclonal antibody DU-HL60-4 (lanes 2, 4, 6, and 8). In panel B, lysates of radioiodinated KG-1a cells (lanes 1 and 2), subclone SJ-67-B (lanes 3 and 4), subclone SJ-67-A (lanes 5 and 6), and control v-*fms* transformed NIH-3T3 cells (lanes 7 and 8) were precipitated with control myeloma protein (lanes 1, 3, 5, and 7) or the MY9 monoclonal antibody (lanes 2, 4, 6, and 8); mol wts were determined by comparison with stained protein standards run on the same gel.

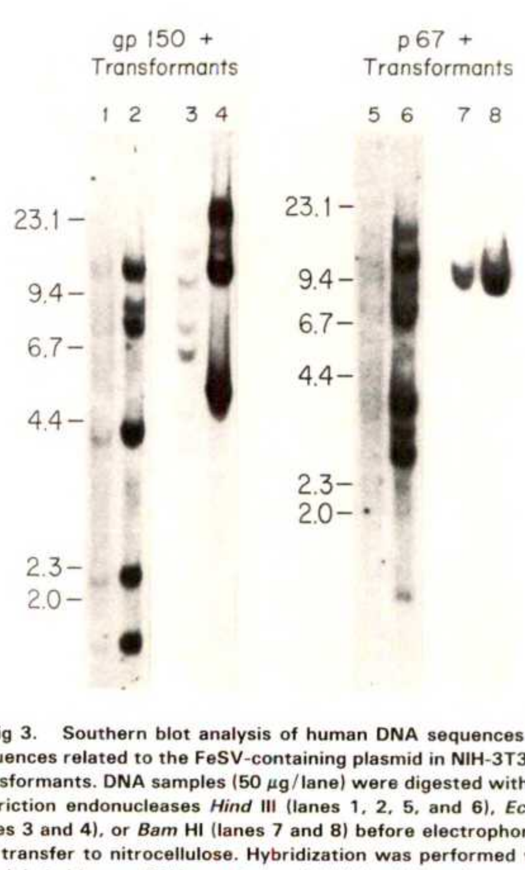

Fig 3. Southern blot analysis of human DNA sequences and sequences related to the FeSV-containing plasmid in NIH-3T3 cell transformants. DNA samples (50 μg/lane) were digested with the restriction endonucleases *Hind* III (lanes 1, 2, 5, and 6), *Eco* RI (lanes 3 and 4), or *Bam* HI (lanes 7 and 8) before electrophoresis and transfer to nitrocellulose. Hybridization was performed with radiolabeled human DNA consisting of highly reiterated sequence families (lanes 1, 2, 5, and 6) or radiolabeled plasmid DNA containing the complete FeSV provirus (lanes 3, 4, 7, and 8). The DNA samples analyzed were from subclones SJ-150-A (lanes 1 and 3), SJ-150-B (lanes 2 and 4), SJ-67-A (lanes 5 and 7), and SJ-67-B (lanes 6 and 8). The positions of bacteriophage λ *Hind* III fragments of known length (noted in kilobase pairs) are indicated to the left of the lanes for each panel.

the sequences encoding gp150 were amplified in SJ-150-B cells. Amplified human DNA restriction fragments were also evident in *Hind* III digests of SJ-67-B DNA, as judged from the intensity of hybridization to similar restriction fragments from SJ-67-A DNA (Fig 3, lanes 5 and 6). As expected, the sizes of the *Hind* III fragments detected with the human repetitive sequence probe differed for transformants selected for expression of gp150 or p67, confirming that these proteins are encoded by different human genes.

DNAs from these subclones were also tested by Southern blotting for amplification of sequences related to the plasmid used as the selectable marker. Nick-translated DNA from a pBR322 vector containing the intact FeSV provirus was hybridized to *Eco* RI digests of DNA from SJ-150-A and SJ-150-B cells (lanes 3 and 4), or to *Bam* HI digests of DNA from SJ-67-A and SJ-67-B cells (lanes 7 and 8). Although each of the subclones expressed similar levels of the v-*fms* gene product on the plasma membrane, amplified sequences related to the FeSV-containing plasmid were evident in DNA from subclone SJ-150-B compared to SJ-150-A (about tenfold amplification) and subclone SJ-67-B compared to SJ-67-A (two- to fourfold amplification).

The heterodisperse pattern of fluorescence observed with the SJ-150-B subclone and monoclonal antibody MY7 (Fig 1A) suggested that human DNA sequences might be unstably amplified in these cells. We therefore attempted to sort gp150-negative cells from the SJ-150-B cell population based on reduced binding with the monoclonal antibody MY7. After three rounds of sorting, a revertant subpopulation (designated SJ-150-BR) that no longer exhibited MY7 binding was obtained. Despite the lack of binding with the gp150-specific antibody, the cells retained the transformed phenotype and continued to show as much binding of the v-*fms*-specific monoclonal antibody SM2.6.3 as that observed with the parental amplified SJ-150-B subclone.

Analysis of *Hind* III-digested DNA from subclones SJ-

150-B and SJ-150-BR after hybridization with probes prepared from both human repetitive DNA sequences and the FeSV-containing plasmid is shown in Fig 4. The characteristic human DNA restriction fragments representing the gp150 gene were considerably more difficult to detect in DNA from subclone SJ-150-BR, as compared with DNA from subclone SJ-150-B (lanes 1 and 2, Fig 4), indicating that gp150 coding sequences had been segregated. Hybridization with the FeSV plasmid probe also yielded a decreased signal intensity in DNA from SJ-150-BR cells as compared with SJ-150-B cells (lanes 3 and 4), although the hybridization intensity was less reduced than that observed with the human repetitive DNA probe. The same restriction fragments were observed in both subclones, reflecting their derivation from a single transformed cell. Therefore, at least some FeSV-containing plasmid sequences can segregate independently of gp150-coding sequences in these clones. Similar experiments were not attempted with subclone SJ-67-B, because the pattern of binding with the monoclonal antibody MY9 was uniformly amplified in these cells with no evidence of a fluorescence-negative subpopulation.

Cytogenetic evidence of gene amplification. Metaphases from transformed NIH-3T3 cell clones were examined for the cytogenetic hallmarks of gene amplification—DM chromatin bodies or ABRs.[11,12] Examination of cells from subclone SJ-150-B with amplified but unstable expression of gp150 showed that 88% of the metaphases contained DMs, at an average of 22 per metaphase (range, 3 to 50). A representative SJ-150-B metaphase spread illustrating multiple DMs per metaphase is shown in Fig 5A. To determine whether DMs were in fact related to overexpression of gp150, we examined metaphases from the SJ-150-BR revertant. These cells contained DMs in 43% of metaphases, with an average of nine DMs per metaphase. Metaphase spreads from subclone SJ-150-A were found to contain DMs at an even lower frequency (17% of metaphases), with an average of only one or two DMs per cell (range, 1 to 5).

The finding of low numbers of DMs in transfected cells lacking evidence of amplified human DNA sequences by Southern blotting led us to examine metaphases from the parental NIH-3T3 line. NIH-3T3 (clone 7) cells were found to contain a low frequency of DMs (10%), with an average of 1.6 per metaphase (range 1 to 3). The presence of distinctive

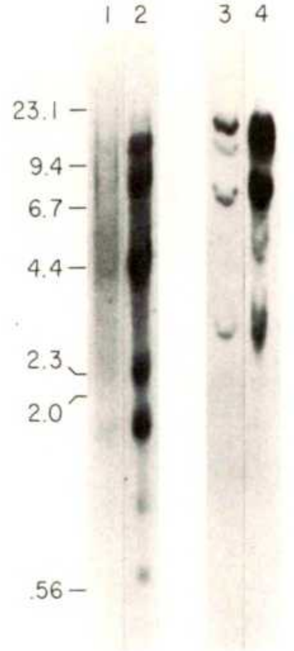

Fig 4. Southern blot analysis of human and FeSV plasmid-related DNA sequences in NIH-3T3 transformants (SJ-150-B) and in revertants (SJ-150-BR) derived from a single clone with unstable amplification of gp150 expression. *Hind* III digests of DNA (50 μg/lane) from subclones SJ-150-BR (lanes 1 and 3) or SJ-150-B (lanes 2 and 4) were hybridized with radiolabeled reiterated human DNA (lanes 1 and 2) or with a plasmid containing the complete FeSV provirus (lanes 3 and 4).

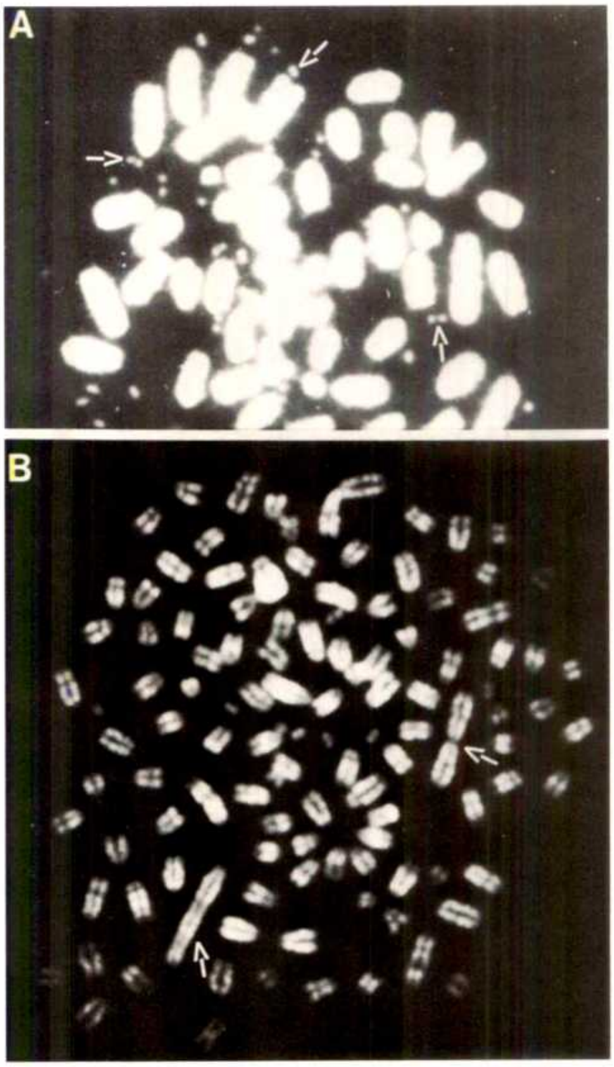

Fig 5. Quinacrine banding analysis of NIH-3T3 transformants SJ-150-B and SJ-67-B. (A): evidence of multiple copies of double minutes (arrows) from the SJ-150-B subclone; (B): demonstration of clonal marker chromosomes in SJ-67-B cells. Arrows indicate a long acrocentric marker chromosome displaying regions of heterochromatic DNA (bottom) as well as the presence of a single large metacentric marker chromosome (top).

marker chromosomes observed in the karyotypes from the transfected NIH-3T3 cells confirm the derivation of these DM-bearing cells from the clone 7 NIH-3T3 parent. Taken together, the results suggest that the expression of gp150 directly correlates with the number and percentage of cells displaying DMs, with high-expressing gp150 clones demonstrating a high number of DMs and low-expressing gp150 clones demonstrating a low number of DMs.

Metaphases from the SJ-67-B subclone displaying a stable pattern of amplified expression of p67 demonstrated extended chromosomal regions of heterochromatic DNA, which stained positively when analyzed with G-, Q-, and C-banding techniques (Figs 5B and 6). Regions of C-band-positive DNA are ordinarily associated with pericentromeric heterochromatin, which usually does not represent a site of active gene transcription. Although DMs were found in cells from subclone SJ-67-A at low frequency (similar to the NIH-3T3 parental cells), they were not detected in any cell from the SJ-67-B subclone.

Localization of human DNA sequences in transfected clones by in situ hybridization. To determine the localization of the human DNA sequences within the mouse genome, we used in situ hybridization.[36] The autoradiographic images of 50 mitotic cells were examined from the subclones SJ-67-B and SJ-150-B following in situ hybridization with ^{3}H-labeled human repetitive DNA.[16] Of the total chromosomal grains localized in SJ-67-B cells, 12% were observed on the near-terminal short arm of an unclassified metacentric marker chromosome (Fig 7). Significant chromosomal localization of the human sequences is consistent with the stable expression of the p67 polypeptide in this subclone. In contrast, in situ hybridization to the DM-bearing SJ-150-B subclone demonstrated a very different pattern of grain distribution, with no significant grain localization to any single mouse chromosome. The lack of a single cytologically recognizable site of chromosomal integration by in situ hybridization, coupled with the aforementioned correlation between the frequency of DMs in SJ-150-B cells and the expression of gp150 protein, make extrachromosomal DMs the likely site of amplified human sequences in this subclone. The size of the DMs in this cell line is essentially identical to the size of the autoradiographic grains following in situ hybridization, making direct localization of human sequences to DMs impossible.

DISCUSSION

DNA-mediated gene transfer into NIH-3T3 cells with the v-*fms* transforming gene as a dominant selectable marker permitted flow cytometric isolation of transformants with amplified expression of human myeloid differentiation antigens. A limited number of human DNA restriction frag-

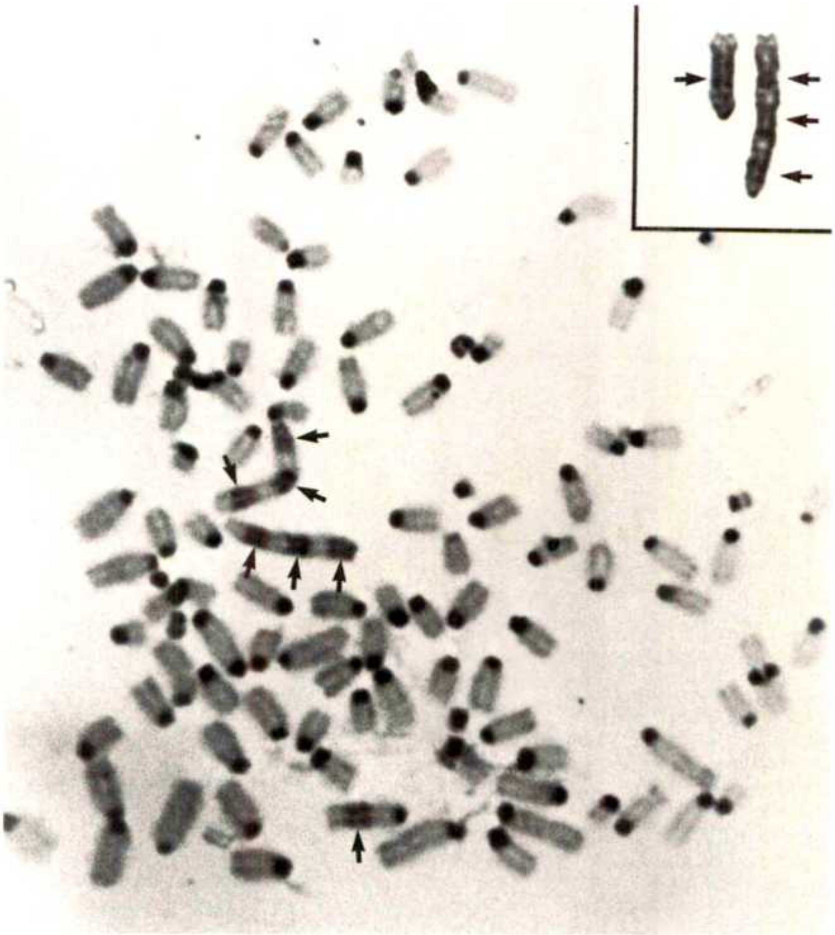

Fig 6. A typical metaphase spread from cloned NIH-3T3 transformants with amplified p67 expression (subclone SJ-67-B). Extended regions of heterochromatic DNA (arrows) were evident in C-banded metaphases. The large acrocentric marker chromosomes may have arisen from fusion of three of the abnormal 1 chromosomes (inset, from a G-banded metaphase).

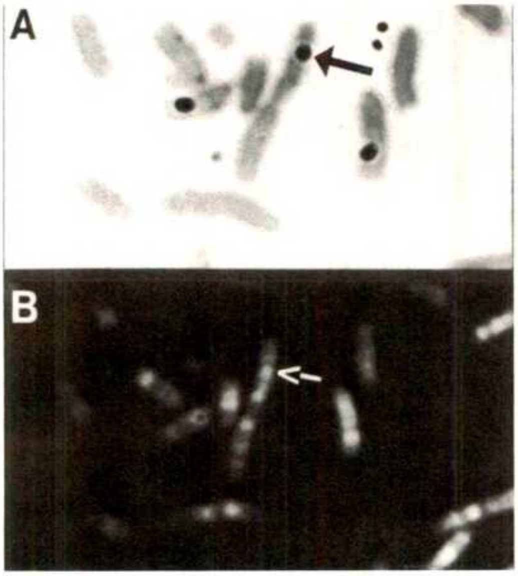

Fig 7. Partial metaphase demonstrating localization of human repetitive DNA utilizing in situ hybridization.[36] (A) Wright's staining demonstrating an autoradiographic grain on the short arm of the large metacentric marker chromosome. Twelve percent of all chromosomal grains localized to this band region. (B) Quinacrine banding prior to in situ hybridization of the same metaphase pictured in panel A, allowing positive identification of the marker.

ments were amplified in cloned secondary or tertiary transformants that exhibited greatly increased expression of the cell membrane antigens, gp150 and p67, as compared with HL-60 donor cells. Transformants with stably amplified genes contained chromosomally integrated donor DNAs, whereas unstable gene amplification was associated with the presence of DMs. By selecting revertant clones, the amplified human DNA sequences in gp150-positive clones segregated with DMs, suggesting that copies of the gp150 gene were associated with these extrachromosomal elements. The failure to localize chromosomal sites of integration in gp150-positive cells was also consistent with the localization of transfected human DNA to DMs. Human DNA sequences did not map to extended heterochromatin regions of p67-positive cells, but rather to an unidentified marker chromosome seen in parental NIH-3T3 cells. Noncentromeric heterochromatic regions analogous to those found in the SJ-67-B subclone have been observed previously and were shown to be associated with increased metastatic potential in Rous sarcoma virus-transformed NIH-3T3 cells.[37] Thus, spontaneous amplification of unidentified mouse sequences in SJ-67-B cells may similarly reflect selection for increased growth potential after FeSV-mediated transformation. As has been found in other systems, amplification of the human DNA sequences in this subclone occurred without a cytogenetically evident ABR at the integration site. The formation of an ABR depends on the size of the amplified unit of DNA rather than on the degree of amplification of sequences encoding a single gene.[4]

Our results with the gp150-positive clones are analogous to those of Kavathas and Herzenberg,[14] who observed amplification of the gene encoding the human T cell membrane antigen Leu-2 (or T8) after co-transfection of human DNA with the cloned herpes simplex *tk* gene into *tk*-deficient mouse L cells. Such transformants provide ideal sources of template RNA for the generation of subtractive cDNA probes to identify genomic or cDNA clones of the transferred gene, as demonstrated for the Leu-2 antigen by these workers[15] and by Littman et al.[38]

The use of v-*fms*-transformed NIH-3T3 cells in DNA-mediated gene transfer experiments offers certain advantages. The transformed cells multiply very rapidly, with shorter intervals between successive rounds of cell sorting, and they can be grown in medium lacking antimetabolites. The c-*fms* proto-oncogene product is a cell surface glycoprotein expressed by cells of the mononuclear phagocyte lineage and is related to the receptor for the macrophage colony-stimulating factor, CSF-1.[39] Although any transforming gene might function as a selectable marker in gene transfer experiments, we cannot exclude the possibility that the v-*fms* gene product in some way facilitates the expression of other myeloid genes. The subclone of NIH-3T3 cells used as recipients[19] may also be particularly suitable for such experiments, in that they contain small numbers of DMs. An attractive possibility is that transfected DNA sequences integrated into DMs in a small proportion of recipient cells, where they underwent spontaneous amplification. In the case of the stably amplified p67-expressing subclones, DMs were no longer detected, and the amplified sequences were chromosomally associated. There are several precedents for cells with DMs and unstably amplified genes to yield subclones with stably amplified genes, suggesting that genetic sequences in the DMs may become stably integrated in chromosomes.[4]

Although the plasmid containing the SM-FeSV provirus was also amplified in some of our clones, the levels of the v-*fms* gene product expressed at the cell surface were not increased. It is possible that the expression of high levels of the transforming glycoproteins is toxic to cells, resulting in a continued selection against their increased expression. Experiments performed with clones expressing gp150 indicated that revertants that segregated gp150 sequences were still morphologically transformed and expressed equivalent amounts of the v-*fms* gene product, even though they had lost copies of the SM-FeSV-containing plasmid. It seems likely, therefore, that at least some FeSV proviral DNA sequences were chromosomally integrated and were sufficient for morphological transformation.

Monoclonal antibodies have been developed that identify epitopes of molecules expressed on the plasma membrane of committed human myeloid progenitors [granulocyte-monocyte colony-forming units in culture (CFU-C)] and subsets of differentiated cells in the granulocyte and monocyte lineages.[26-29,32] The gp150 molecule is expressed by a proliferative subset of human CFU-C, as well as on normal marrow granulocytes and monocytes, at all recognizable levels of differentiation. Antibodies specific for gp150 do not bind normal B or T lymphocytes, platelets, erythrocytes, or erythroid progenitors defined by burst-forming units (BFU-E) or colony-forming units (CFU-E) in culture.[26,27]

The previously uncharacterized surface molecule immunoprecipitated by MY9 has an apparent mol wt of 67 kd. Although characterization of the molecule has been hampered by its relatively low levels of expression in human myeloid cells, transformants with amplified p67 expressed readily detectable levels of the polypeptide. High levels of fluorescence in flow cytometric studies were also observed after binding of the independently derived monoclonal antibodies L4F3 and L1B2,[32] providing evidence that these three antibodies recognize epitopes represented on the product of a single human gene. Antibodies specific for p67 bind all CFU-C and subsets of BFU-E and multipotent progenitors (CFU-GEMM), as well as myeloblasts, promyelocytes, myelocytes, and circulating monocytes. Binding is weak or absent on mature granulocytes, and not detected on lymphocytes, erythrocytes, and platelets.[28,32]

Specific antibodies to gp150 or p67 bind malignant cells from most patients with myeloid leukemias, but do not bind to leukemic cells of lymphoid origin.[26,28] This has led to applications of these antibodies in clinical diagnosis. Griffin et al, for example, have reported that 93% of myeloid leukemias can be identified from the binding of MY7 or MY9 antibodies.[28] Despite extensive studies of the distribution of gp150 and p67 on normal and malignant human myeloid cells, the functions of these surface molecules are unknown. It is clear that DNA-mediated gene transfer leading to gene amplification in transformed subclones should facilitate further characterization of these genes and their products and should provide a general method for isolating genes that encode a variety of cell-surface molecules.

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Erythropoietin Kinetics in Rats: Generation and Clearance

By Stephen E. Steinberg, Joseph F. Garcia, Gary R. Matzke, and Jeanette Mladenovic

Detailed studies to analyze the early events of erythropoietin (Ep) secretion and clearance were performed in a rat model using a double antibody radioimmunoassay. Ep clearance was determined following intravenous injection of 1 mL of Ep-rich plasma, 1,080 mU/mL, obtained from phlebotomized rats. Analysis revealed a disappearance curve that conformed to a two-compartment model with an α half-life t ½ of 3.6 minutes and a β t ½ of 86 minutes. The volume of distribution was similar to the calculated plasma volume. In anephric animals, there was no change in the plasma clearance rate or the volume of distribution. Rapid Ep secretion was elicited by a single 15 mL/kg phlebotomy (hematocrit decrement 45% to 30%), so that levels reached 20 to 30 times baseline (524 ± 76 *v* 24 ± 7 mU/mL) at five hours, whereas they plateaued for at least 33 hours. The increase in the rate of secretion was geometric, from 9.9 mU/h baseline secretion to 429 mU/h. These data identify a very sensitive and rapidly responsive system for Ep modulation in the rat.

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ERYTHROPOIETIN (Ep), a glycoprotein hormone of renal origin, stimulates red cell proliferation and differentiation in mammals in response to hypoxia.[1] As studies begin to demonstrate the potential therapeutic usefulness of this hormone in such disease states as chronic renal failure, an understanding of its pharmacokinetic properties becomes important.[2] In the past, detailed studies of Ep secretion and clearance have been limited by the lack of an assay sensitive enough to detect small changes in hormone levels.[3] The recent development and availability of a radioimmunoassay (RIA) for Ep have made these studies feasible.[4] We used the RIA in a rat model to determine the clearance rate of homologous Ep and to describe the early events of Ep secretion.

MATERIALS AND METHODS

Sprague Dawley rats, weighing 200 to 250 g, were used in all experiments. The source of Ep for clearance studies was plasma obtained from donor rats made severely anemic by every-other-day phlebotomy until the hematocrit reached 12% to 18%. The animals were then exsanguinated by aortic puncture, the heparinized blood was centrifuged at 2,500 rpm for 30 minutes, and the plasma was pooled. This Ep-rich plasma, although impure, represented a physiological form of the hormone that would be subject to endogenous clearance. Ep activity was assayed by the double-antibody RIA using a rat standard curve.[4,5] The intra-assay coefficient of variation was 8.4%. The Ep titer of the plasma used for all studies was 1,080 mU/ml. The bioactivity of the pooled Ep was demonstrated by intravenous (IV) injection of 1.0 mL into three animals followed by twice-daily determinations of the reticulocyte response. This resulted in a rise in reticulocyte count from a baseline of 0.6% ± 0.2% (SEM) to 5.8% ± 1.2% on day 3.

For clearance studies, animals were anesthetized with Innovar-Vet, and a polyethylene catheter was placed in the right atrium through the jugular vein for vascular access. One milliliter of Ep-rich plasma was given as an IV bolus. Serial 0.5-mL samples of heparinized blood were obtained through the central venous catheter from 30 seconds to two hours. Following each sample, volume was replaced with normal donor blood. Separated plasma samples were frozen at −20 °C until assayed by RIA. To study the effect of renal clearance on Ep disappearance, animals were anesthetized with Innovar-Vet and nephrectomized immediately before Ep-rich plasma was injected. This approach minimized volume changes related to diminished renal function.

To investigate Ep secretion, 12 animals underwent a 3-mL phlebotomy (15 mL/kg) after insertion of a central venous catheter as above. Immediate volume replacement with pooled plasma from normal donor animals (Ep = 18.5 ± 2.5 [SEM] mU/mL) resulted in a fall in hematocrit from an initial mean value of 45 ± 2.8 (SEM) to 29.5% ± 3.1%. Serial samples were again removed at intervals for Ep determination. An equivalent volume of normal donor blood adjusted to an hematocrit of 30 was reinstilled after each sampling to avoid hemodilution and hypovolemia.

Analyses. The plasma clearance data were analyzed by the following equation based on minimization of the residual sum of squares:

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t}, \qquad [1]$$

where $C(t)$ is the concentration in serum at time t; A and B are the y intercepts, and α and β are the disposition rate constants obtained from the first and second phases of the plot of log serum Ep concentration *v* time. Initial estimates of the kinetic parameters were generated by standard curve stripping techniques.[6] Final estimates were obtained by nonlinear regression analysis with the program KINA on a Control Data digital computer (University of Minnesota, Minneapolis). The volume of distribution of the central compartment, the steady-state volume of distribution, the area under the serum concentration curve from zero to infinity, and the total body clearance were calculated by standard techniques.[6]

Stimulated secretion data were analyzed as follows. The baseline Ep serum concentration data were used to determine the unstimulated secretion rate (Ep Base) in 12 animals. The baseline Ep concentrations were assumed to reflect steady-state conditions, with the amount of Ep formed per unit of time equal to the amount of Ep removed during the same period of time. The concentration time curve from 0 to six hours was then analyzed on a Hewlett-Packard 9845 desktop computer by linear regression analysis and also by polynomial regression analysis from 0 to 60 hours [Equation 2]. The maximal Ep secretion rate (Ep max) was estimated from the ratio of the stimulated plateau Ep concentration (C_{STIM}) to baseline Ep concentration (C_{BASE}), assuming that total body clearance remained constant [Equation 3].

$$C(t) = m_1(t) + m_2(t^2) + b \qquad [2]$$

$$\text{Ep max} = [C_{\mathrm{STIM}}/C_{\mathrm{BASE}}] \cdot \text{Ep base} \qquad [3]$$

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RESULTS

Erythropoietin clearance following IV bolus of 1 mL of Ep-rich plasma (1,080 mU) conformed to a two-compartment model (Fig 1 and Table 1). An initial rapid α disappearance, t½ = 3.6 minutes, was followed by a longer β phase, t½ = 86.4 minutes ($r = 0.97$, $P < .01$). The steady-state volume of distribution (4.27 mL) was similar to the plasma volume of these rats (5.1 mL).[7]

When the clearance study was repeated in anephric animals, similar results were obtained (Fig 2 and Table 1). Again the data conformed to a two-compartment model. When compared with clearance in normals, Ep clearance in anephric animals had an α phase that was several minutes longer, although the difference was not statistically significant. Although the β disappearance was prolonged due to the increased volume of distribution, total body clearance for anephric animals was slightly greater than normal, but the difference was not statistically significant (Table 1).

The early events of erythropoietin secretion are shown in Fig 3. In response to a single 15-mL/kg phlebotomy, a 21-fold increase in Ep levels was observed, from a baseline of 21 mU/mL to 525 mU/mL. Levels increased within minutes, began to plateau at five hours, and remained elevated for at least 33 hours. Using the homologous clearance data generated in the first portion of this study, the rate of secretion required to maintain the normal Ep level of ~20 mU/mL was calculated to be 53 mU/h. In response to a single phlebotomy, this basal rate of secretion doubled approximately every hour until it reached 450 mU/h at plateau.

DISCUSSION

These experiments detail the kinetics of Ep secretion and clearance, using homologous hormone, assaying levels by a sensitive RIA, and incorporating concurrent clearance data into a secretion model and vice versa. Following the bolus administration of EP-rich plasma, a two-phase clearance curve was obtained, as with previous studies.[8-11] Although studies using nonhomologous and/or biochemically altered Ep have yielded a slow equilibrium phase (ascribed to a large Ep molecule dispersing into a sizable extravascular space),[8,11] in this study the mean distribution phase t½ was 3.6 minutes. This rapid distributional phase might be expected in view of the volume of distribution, which was equivalent to the plasma volume of the animal.[7] This result is consistent with studies in sheep, also using homologous Ep, in which the Ep space was the same as the measured plasma volume.[12] In contrast, using a heterologous protein (sheep Ep injected into rats), Reissmann found that the volume of distribution approximated twice the plasma volume[8] It is possible that the initial rapid clearance of a foreign protein or of Ep bound to a heterologous carrier protein could yield an inordinately large volume of distribution, perhaps accounting for the discrepancy.[11]

Table 1. Erythropoietin (Ep) Clearance Data Following Intravenous Bolus of 1 mL of Ep-Rich Plasma

Clearance	Normal (Mean ± SEM)	Anephric (Mean ± SEM)	*P* Value
α t (min)	3.62 ± 0.51	7.80 ± 4.4	NS
β t (min)	86 ± 16	111 ± 21	NS
V_1 (mL)	1.87 ± 0.19	2.55 ± 0.45	NS
V_{ss} (mL)	4.27 ± 0.29	4.78 ± 0.23	NS
Total body (mL/min)	2.3 ± 0.31	2.48 ± 0.89	NS

Clearance data for 1 mL (1,080 mU) of erythropoietin in normal and anephric animals V_1, central compartment volume of distribution; V_{ss}, steady state volume of distribution.

Following the distribution phase, the second phase, that of plasma clearance, was linear from ten minutes to the end of the two-hour assay period, with a t½ of 86 minutes. Naets and Wittek, also using a biologic assay and homologous, exogenous Ep,[13] obtained a t½ of 1.5 hours Slightly longer t½s have been obtained in studies of Ep disappearance following the withdrawal of a hypoxic stimulus.[8,13,14] This difference may be due to ongoing Ep secretion, which persists even after withdrawal of the stimulus, thereby prolonging the apparent t½.[8] Other clearance data have been published, with Ep t½s ≤24 hours.[8-12,14-21] In addition to species variability,[10,12,15-17] there are several methodological differences between these studies and the present one. In some studies, the use of nonhomologous and/or iodinated serum Ep or even urinary Ep could have resulted in nonphysiologic clearances of "foreign" proteins or Ep aggregates.[8-11,15,16] As suggested by Emmanoel et al, it is possible that heterologous ^{125}I-Ep is metabolized differently than endogenous Ep, reflecting either the heterologous nature of the protein or the possibility that the iodination process, which renders the protein biologically inactive, also alters its clearance characteristics.[11] Although the use of ^{125}I-labeled Ep obviates confusion in the data due to ongoing endogenous production of the hormone,[11] this difficulty may also be resolved by incorporating the experimentally determined secretory rate into the calculations of Ep clearance.

There has been controversy regarding the role of the kidney in Ep clearance.[3] Early studies demonstrated that

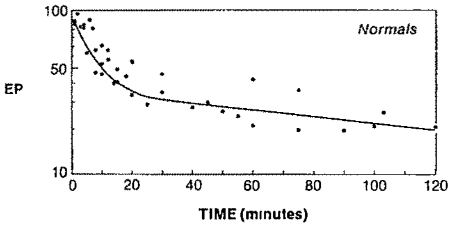

Fig 1. Clearance data following intravenous bolus of homologous Ep (1,080 mU) into ten normal rats.

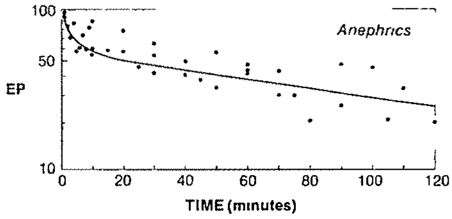

Fig 2. Clearance data following intravenous bolus of homologous Ep (1,080 mU) into ten anephric rats.

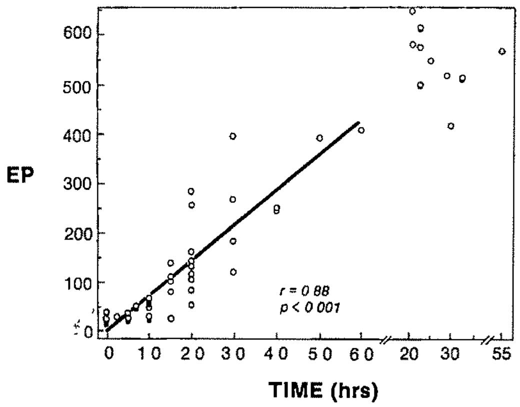

Fig 3. Ep levels (mU/mL) following 15-mL/kg phlebotomy in 12 rats.

urinary excretion parallels plasma concentration, but that urinary excretion constituted only a small portion of clearance.[10] Subsequent studies, nevertheless, have reported prolongation of Ep clearance after nephrectomy.[11,13-14,22,23] Recently, Emmanoel et al, using a highly purified erythropoietin, reported a very slight but statistically significant contribution of the kidneys to total body clearance [11] In the current study, nephrectomized animals had a slight, statistically insignificant prolongation of the plasma t½ and no change in the total body clearance. Mladenovic et al found no difference in Ep clearance in sheep both prenephrectomy and postnephrectomy.[12] It seems likely that the renal contribution to Ep clearance is less than 10%. It is possible that the loss of a renal contribution to Ep clearance and/or catabolism results in a compensatory increase in Ep clearance by other tissues.

In the past, it has not been possible to determine the rate of Ep secretion because of the requirement for accurate basal levels and the incorporation of plasma disappearance data.[3] In this study, the availability of the RIA and homologous plasma clearance data have allowed an accurate determination of the kinetics of Ep production. The normal Ep level of 19 mU/mL was maintained by a basal Ep secretory rate of 53 mU/h. When the animals were rapidly rendered anemic, the rate of Ep production increased geometrically, to at least nine times baseline levels. The Ep level rose almost instantaneously, reached a plateau at five to six hours and remained at this elevated level for up to 33 hours, suggesting persistent Ep secretion. The continuing Ep secretory response to the experimentally-induced anemia in this study may correspond to the clinical situation in which elevation of the Ep level is proportional to the severity of the anemia.[1] Although some studies of the time course of the Ep response are consistent with our data,[25,26] others have shown an early peak and rapid falloff of Ep levels by 24 hours.[24,25,27,28] These differences may be partially explained by differences in the various methods used to induce hypoxic stress (eg, phlebotomy, phenylhydrazine-induced anemia, hypoxia, and iron deficiency anemia). Miller et al and Cohen et al have further suggested that acid-base disturbances associated with both hypoxia and volume depletion could play a role in the early fall in Ep levels after stimulation.[27-29]

This study demonstrates the existence of a very sensitive system for Ep modulation in the rat. The immediate secretory response to anemic stress and the limitation of distribution to the plasma compartment work to initiate a rapid bone marrow response to changes in oxygen delivery. The relatively slow rate of clearance maintains stimulation of a slowly responsive end organ.

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Cytogenetic and Immunophenotypic Analysis of Cell Lines Established From Patients With T Cell Leukemia/Lymphoma

By Stephen D. Smith, Rodman Morgan, Michael P. Link, Pamela McFall, and Frederick Hecht

Cell lines were established from five patients with T cell malignancies. Two patients had T cell lymphoblastic lymphoma (T-LL), whereas three patients had T cell acute lymphoblastic leukemia (T-ALL). Both T-LL cell lines expressed cell surface antigens characteristic of mid-thymocytes (Leu 2, 3, 6+). One T-ALL cell line also expressed this immunophenotype, one expressed suppressor/cytotoxic antigens (Leu 2+; Leu 3, 6−), and one expressed antigens of a mature but uncommitted T cell (Leu 4+; Leu 2, 3, 6−). Cytogenetic analysis showed that each cell line had 46 chromosomes with pseudodiploidy. The three T-ALL cell lines had only a few chromosome changes; one cell line had one deletion, another had two deletions, and the third had a translocation and two deletions (including loss of part of 9p). In comparison, both T-LL cell lines had complex chromosome changes, including most notably a rearrangement of band 14q11.2. The immunophenotypes and chromosome breakpoints showed patterns of interlock between the T-LL and T-ALL cell lines because common abnormalities occurred at six distinct chromosome sites. Cell lines with limited and specific chromosomal abnormalities are important because they can provide the basic material for molecular genetic studies that could elucidate the genetic mechanisms involved in neoplasia.

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T-CELL LYMPHOID malignancies are a heterogenous group of diseases which express cell surface markers characteristic of thymus tissue In children and in young adults, two clinically similar T cell malignancies predominate: T cell acute lymphoblastic leukemia (T-ALL) and T cell lymphoblastic lymphoma (T-LL).[1,2] The T cell phenotype is seen in ~15% of cases of acute lymphoblastic leukemia, and patients initially often have a peripheral white blood count <100,000/μL, and a mediastinal mass. T-ALL shares some clinical features with T-LL; both diseases are associated with a mediastinal mass, are more common in males, and occur more commonly in adolescents and young adults.[1-3]

T-LL is arbitrarily distinguished from T-ALL by the lack of extensive bone marrow involvement (<25% marrow blasts).[4] Patients with T-LL generally have a more mature immunophenotype and a longer survival than do patients with T-ALL.[5-9] These distinctions suggest that the differences between these two diseases may be more than the degree of dissemination present at diagnosis.

It may be possible to separate T-cell malignancies more completely by chromosome analysis. Although karyotypes have been reported in >100 T cell malignancies, most cases have been found in patients with Japanese T cell leukemia or cutaneous T cell lymphoma.[10-13] In addition, only a few karyotypes have been reported from children with T-LL and T-ALL.[14] We recently established cell lines from two patients with T-LL and three patients with T-ALL. Both T-LL cell lines had complex chromosome changes that included a rearrangement of band 14q11.2. In contrast, the T-ALL cell lines had only a few chromosomal changes, yet abnormalities common to both T-ALL and T-LL were observed.

MATERIALS AND METHODS

Source of malignant cells The data discussed in this report were derived from five patients with T-LL or T-ALL. The protocol procedures were approved by the Medical Committee for the Use of Human Subjects in Research at Stanford University and informed consent was obtained from the parents and/or patients

The clinical and pathologic data on each patient are presented in Table 1 At initial examination, each patient had a large mediastinal mass, and the two patients with T-LL had a normal bone marrow aspirate and biopsy The patients with T-ALL had bone marrow involvement (M_3 bone marrow), and each had a white blood count >100,000/μL (with >80% circulating lymphoblasts) Malignant cells were collected from pleural effusion, bone marrow, or peripheral blood, and were anticoagulated with preservative-free heparin. Within four hours of collection, malignant lymphoid cells were separated by Ficoll-Hypaque density sedimentation and washed with "complete media" consisting of McCoy's 5A media supplemented with 15% fetal calf serum (FCS), penicillin (50 U/mL) and streptomycin (50 μg/mL) After a second washing, the malignant cells were separated into aliquots for cell culture experiments and immunophenotypic analysis.

Establishment and maintenance of cell lines The technique for culturing malignant T cells was a modification of our previously reported methods.[15,16] In brief, separated malignant cells were warmed to 37 °C in complete media and were pipetted to break up cell aggregates. The resultant single-cell suspension next was mixed with agar (0.3%) and plated onto 35-mm Petri dishes containing a feeder layer consisting of a combination of complete media, normal human serum (10%), and agar (0 5%) The Petri dishes were incubated at 37 °C in a Heraeus incubator gassed with 5% O_2, 6% CO_2, and 89% N_2. After 18 days of growth, individual colonies were aspirated from the agar, suspended in complete media (without agar), and transferred to Petri dishes containing a freshly prepared feeder layer. Cells were observed every three to five days and were passaged when the media appeared acidic (ie, when the phenolphthalein indicator turned yellow), or when the cell number approached 5 × 10^5 cells per milliliter. It was of particular importance that throughout the complete incubation period the cell culture

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Table 1. Clinical Data on Five Patients With Malignant T Cell Disease

Age/Sex	Diagnosis	Clinical Status	Sample Cultured	Cell Line Established
16 yr/M	T-ALL	Relapse	BM	K-T1
52 yr/F	T-ALL	At diagnosis	BM	SUP-T2
12 yr/M	T-ALL	Relapse	PB	SUP-T3
8 yr/M	T-LL	Relapse	PE	SUP-T1
24 yr/M	T-LL	Relapse	PE	SUP-T4

T-ALL, T cell acute lymphoblastic leukemia; T-LL, T cell lymphoblastic lymphoma, BM, bone marrow; PE, pleural effusion; PB, peripheral blood.

system contained no added mitogens, thiols, conditioned media, or interleukins.

Once steady cell growth occurred on the Petri dishes, cells (5×10^5 cells per milliliter) were passaged into flasks and thereafter grown in suspension culture in complete media. Initially, the flask contained a feeder layer; the volume of the feeder layer subsequently was reduced if cell viability could be maintained. Once the cells grew without a feeder layer, the oxygen concentration was gradually increased over a period of weeks until the cells had adapted to growth in an environment of 5% CO_2 in air. Each T cell line grew to a saturation concentration of $\sim 1 \times 10^6$ cells per milliliter and was routinely split three times per week. The average doubling of cells in log phase growth was calculated from daily counts. The cloning efficiency of the T cell lines was determined by mixing cells (1×10^5 cells per milliliter) with agar (0.3%) and replating them into a Petri dish containing a feeder layer. After 18 days of growth, colonies (cell groups containing >50 cells) were counted, and the cloning efficiency (CE) was calculated: CE = (number of colonies grown ÷ number of cells plated) × 100.

Characterization of cell surface antigens. Monoclonal antibodies to Leu-1 (Pan T), Leu-2a (T cytotoxic/suppressor), Leu-3a (T helper), Leu-4 (Pan T), Leu 5 (sheep erythrocyte receptor), Leu 6 (thymocyte), Leu 9 (Pan T), CALLA (common ALL antigen), and HLA-DR were generously provided by the Becton Dickinson Corporation (Mountain View, Calif). OKT_9 and OKT_{10} were purchased from Ortho Pharmaceuticals (Raritan, NJ). Surface immunoglobulin was detected by the $F(AB')_2$ fragment of fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin (TAGO, Burlingame, Calif). Cell surface antigens were identified by the binding of monoclonal antibody as detected by indirect immunofluorescence according to established methods.[17] Cells were analyzed for fluorescence staining with a FACS IV (Becton Dickinson Electronics Laboratory). The results were expressed as percentage of positive cells compared to background fluorescence when nonreactive myeloma protein was used in place of specific antibody.

Karyotype analysis. One drop of colchicine (Colcemid, GIBCO, Chagrin Falls, Ohio; 10 μg/mL in phosphate-buffered saline) was added to cells (log phase growth, 3×10^5 cells per milliliter) and incubated (37 °C, 10% CO_2 in air) for ½ to 1½ hours. The cells were pelleted by centrifugation, resuspended in 0.075 mol/L of KCl for ten minutes, fixed in fresh methanol-glacial acetic acid (3:1), and centrifuged. The cells were resuspended in fixative and left overnight at 4 °C. The next day, the cells were dropped onto glass slides and air dried, and the chromosomes were G-banded.[18] From each culture, at least 20 cells were analyzed microscopically, and karyotypes were prepared.

RESULTS

The clinical data on the five patients with T cell malignancies are shown in Table 1. These patients ranged in age from 8 to 52 years, and each patient had a large mediastinal mass when first examined. Cell lines were established from pleural effusions from the two patients with T-LL and from blood or bone marrow from three patients with T-ALL. One cell line (SUP-T2) was established from a patient at first examination (prior to therapy); the other four cell lines were established from patients at relapse.

The immunophenotyping studies of the T cell lines are listed in Table 2. Each cell line expressed pan-T markers (Leu 1, Leu 9) and at least two other T cell-specific antigens. In addition, each cell line was nonreactive with monoclonal antibodies against CALLA and HLA-DR and failed to express SIg. The two T-LL cell lines (SUP-T1, SUP-T4) and one of the T-ALL cell lines (SUP-T3) had cell surface antigens characteristic of the midthymocyte maturation stage.[7] These cell lines expressed Leu 2, Leu 3, and Leu 6 on the cell surface but lacked Leu 4. The SUP-T2 cell line had the cell surface characteristics of a suppressor/cytotoxic T-cell (Leu 2+, Leu 3−, Leu 6−). The K-T1 cell line was established in culture in 1978, and immunophenotyping on this cell line was first performed in 1982. The K-T1 cell line was Leu 4+, Leu 2−, Leu 3−, Leu 6−, indicating a mature but uncommitted immune profile. The antigen profile on the cell lines has been evaluated every six months, and it has remained stable without antigen gain or loss.

The growth characteristics of the five T cell lines are shown in Table 3. The T-LL cell lines (SUP-T1, SUP-T4) had a more rapid doubling time in suspension culture and a higher cloning efficiency in agar than did the T-ALL cell lines. The SUP-T2 cell line divided slowly in suspension culture and did not form colonies when resuspended in agar. The addition of supplemental sera, nutrients, and T cell growth factors did not enhance the cellular proliferation of the SUP-T2 cells.

The results of the cytogenetic analysis of the five T cell lines are shown in Table 4. Each cell line except cell line K-T1 was examined within the first six months of culture. When chromosome analysis without G-banding was initially performed on the K-T1 cell line, the translocation and deletions were not detected.[16] The T-ALL cell lines (K-T1, SUP-T2, SUP-T3) had comparatively few chromosomal changes whereas the T-LL cell lines (SUP-T1, SUP-T4) had a considerable number of complex chromosome aberrations.

Table 2. Immunophenotype of T Lymphocyte Lines

	T-ALL			T-LL	
Marker	K-T1	SUP-T2	SUP-T3	SUP-T1	SUP-T4
β_2M	+	+	+	+	+
Leu 1	+	+	+	+	+
Leu 2	−	+	+	+	+
Leu 3	−	−	+	+	+
Leu 4	+	−	−	−	+
Leu 5	+	+	+	−	+
Leu 6	−	−	+	+	+
Leu 9	+	+	+	+	+
OKT9	+	+	−	−	+
OKT10	+	−	+	+	+

−, <20% reactivity; +, >20% reactivity. Each cell line was nonreactive with SIg, CALLA, and HLA-DR.

Table 3. Growth Characteristics of Established T Cell Lines

Cell Line	Cell Type	Doubling Time (h)	Cloning Efficiency (%)†
K-T1	T-ALL	68	9
SUP-T2	T-ALL	130	0
SUP-T3	T-ALL	61	2
SUP-T1	T-LL	28	40
SUP-T4	T-LL	33	38

*Doubling time calculated from cells in log phase growth in liquid culture.

†Cloning efficiency = (no. of colonies grown in agar ÷ no. of cells plated) × 100.

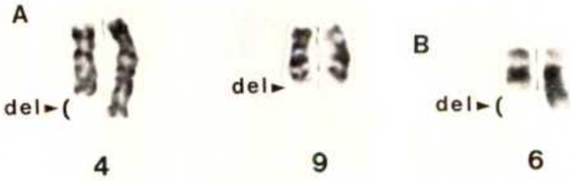

Fig 1. (A) Selected G-banded chromosomes depicting consistent chromosome defects found in cell line SUP-T3. Arrows indicate the site of del(4)(q31q35) and del(9)(q32q34). (B) Partial karyotype of cell line SUP-T2 analyzed by G banding. Arrow indicates the site of del(6)(q21q27).

The T-ALL cell line, SUP-T2, had a single chromosomal abnormality del 6 (q21q27), whereas the SUP-T3 cell line had two terminal deletions: del 4 (q31q35) and del 9 (q32q34) (Fig 1). These abnormalities were stable on repeat analysis and were the only abnormalities observed. The K-T1 cell line had an interstitial deletion on chromosome 2 del 2 (p16.3p22.1) and on chromosome 9 del 9 (p12p21.1) as well as a single translocation t(1;11) (p36.2;p13) (Fig 2). In addition, the K-T1 cell showed some signs of clonal evolution: a few cells lacked a chromosome 20 or gained a chromosome 11.

In comparison, the NHL cell line SUP-T1 was predominantly tetraploid and had multiple inversions and deletions. This cell line possessed two copies of an inversion on chromosome 2, inv(2) (q11p21), and a translocation involving the distal end of the long arm of chromosome 9, t(9;?) (q34;?); no normal copies of chromosome 2 or 9 were observed (Fig 3). The NHL cell line SUP-T4 had extensive chromosome numerical instability and a complex pattern of translocations, chromosomal loss, and marker chromosomes (Table 4, Fig 4).

Some of the chromosomal breakpoints in the T cell lines were common to more than one cell line (Table 5). One NHL cell line (SUP-T1) possessed changes that were found in each of the four other T cell lines. Both T-LL cell lines had a change at chromosome band 14q11.2. The SUP-T1 cell line had an inversion involving chromosome 14, inv(14) (q11.2q32), whereas the SUP-T4 cell line had a translocation involving the same band on chromosome 14, t(10,14) (q23;q11.2).

The SUP-T3 cell line, a mid-thymocyte cell like SUP-T1 and SUP-T4, had two chromosome abnormalities in common with SUP-T1. Both cell lines had a chromosome 4 deletion, del 4 (q31q35), as well as an abnormality at band q34 on chromosome 9. The SUP-T2 cell line had a single karyotype abnormality, del 6 (q21q27), and it shared the loss of chromosome 6 (q23q27) with the SUP-T1 cell line. Both the

Table 4. Cytogenetic Analysis on T Cell ALL/NHL Cell Lines

1. T-ALL
   Cell Line: K-T1
   a. Karyotype 46,XY,t(1;11),del(2),del(9)
   b. Breakpoints in chromosome rearrangements: t(1;11), (p36.2;p13), del(2)(p16.3p22.1), del(9)(p12p21.1)
   Cell Line: SUP-T2
   a. Karyotype 46,XX,del(6)
   b. Breakpoints in chromosome rearrangement: del(6)(q21q27)
   Cell Line: SUP-T3
   a. Karyotype 46,XY,del(4q),del(9q)
   b. Breakpoints in chromosome rearrangements: del(4)(q31q35), del(9)(q32q34)
2. T-LL
   Cell Line: SUP-T1*
   a. Karyotype 46,XY,inv(2),inv(2),del(4q),del(6q),del(7q),t(9;?), t(9;?),inv(14)
   b. Breakpoints in chromosome rearrangements: inv(2)(q11p21) two copies, del(4)(q31q35), del(6)(q23q27), del(7)(q32q36), t(9;?)(q34;?) two copies, inv(14)(q11.2q32)
   Cell Line: SUP-T4†
   a. Representative karyotype 46,XY,−9,+der(9)t(9;16), t(10;14),−18,−19,−20,+der(20),+mar,+mar
   b. Breakpoints in chromosome rearrangements: t(9;16) (qter→q12::q13→pter), t(10;14) (q23;q11.2), der(20)t(1;20) (pter→p22::q13→pter)
   c. Other rearrangements: mar, origin unknown; mar, origin unknown

*Mostly tetraploidy in culture.

†Extensive chromosome numerical instability.

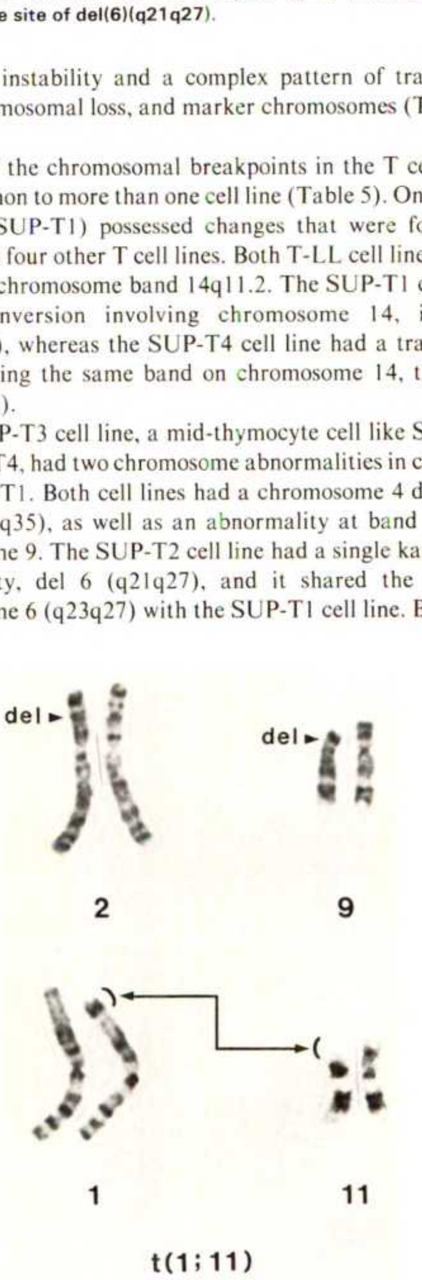

Fig 2. Selected G-banded chromosomes depicting consistent chromosome defects found in cell line K-T1. Arrows indicate the site of del(2)(p16.3p22.1), del(9)(p12p21.1), and a translocation between chromosome 1 and chromosome 11—t(1;11)(p36.2; p13).

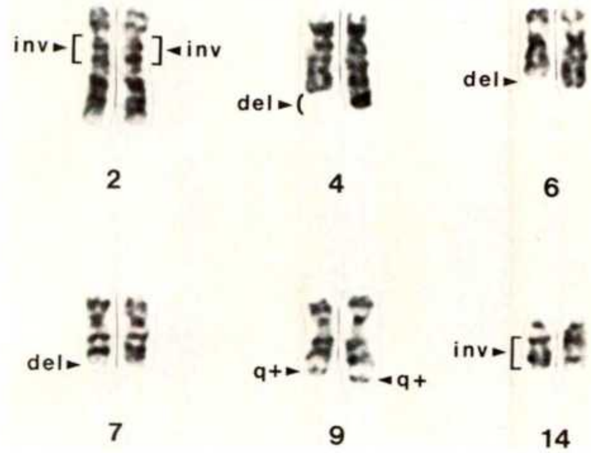

Figure 3. Selected G-banded chromosomes depicting consistent chromosome defects found in cell line SUP-T1. Arrows indicate the sites of inv(2)(q11p21), del(4)(q31q35), del(6) (q23q27), del(7)(q32q36), t(9;?)(q34;?), and inv 14 (q11.2q32).

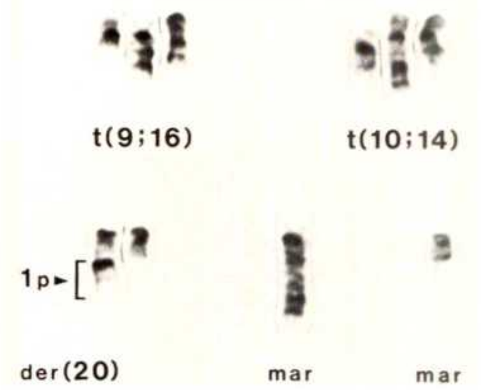

Fig 4. Selected G-banded chromosomes depicting consistent chromosome defects found in cell line SUP-T4. Arrows indicate the site of t(9;16)(qter → q12::q13 → pter), t(10;14)(q23;q11.2), der(20)t(1;20)(pter → p22::q13 → pter) and two marker (mar) chromosomes.

SUP-T1 and K-T1 cell lines had rearrangements involving proximal sites on chromosome 2. The SUP-T1 cell line had two copies of inv 2 (q11p21), whereas the K-T1 had a deletion involving chromosome 2, del 2 (p16.3p22.1). The T-LL cell line, SUP-T4, also shared a chromosome 9 abnormality with the K-T1 cell line. Both cell lines lacked chromosome 9 region p12 → p21.2 because the K-T1 had a chromosome 9 deletion, del 9 (p12p21.1), whereas the SUP-T4 cells had a translocation, t(9;16) (qter → q12::q13 → qter), resulting in the loss of the entire 9p arm.

DISCUSSION

Cell lines have been established from five patients with acute T cell malignancies; each cell line possessed specific chromosomal abnormalities. Although cytogenetic analyses were not performed on the patients' malignant cells, several lines of evidence suggest that the karyotype changes in the cell lines are representative of the chromosomal abnormalities in the patients' original malignant cells. First, prior to cell culture, the patients' malignant cells (in four of five cases) were characterized by a panel of monoclonal antibodies and biochemical enzyme markers.[19] The immunophenotype and biochemical enzyme profile of the patient's malignant cells were compared to the cell line established from an aliquot of these cells. The monoclonal antibody profile of the SUP-T1→T4 cell lines had the same basic immunophenotype as the patient's lymphoblasts, and full concordance existed for 48 of 52 paired antibody tests performed. In addition, each cell line had the same level of elevation of adenosine deaminase and reduction of nucleoside phosphorylase as the patient's malignant cells.[19]

Second, using DNA probes specific for the T cell receptor, each cell line had a rearrangement of the T cell receptor genes with respect to the germ line configuration (D. Faller and S.D. Smith, personal communication). The cell lines appeared unique and clonal, because each cell line had a different rearrangement pattern and no extra bands appeared on the gels. Third, the cell lines (except SUP-T4, which had chromosomal number instability) were chromosomally stable and specific karyotype abnormalities appeared in each cell analyzed. Finally, the karyotype of the cell lines established in our laboratory from patients with Burkitt's lymphoma or common ALL have good chromosomal concordance with patient's malignant cells analyzed prior to culture.

Karyotypic analyses of the T-cell lines revealed chromosomal breakpoints that were common to more than one cell line (Table 5). Six chromosome regions possibly are nonrandomly involved in T cell malignancies: 14q11.2; 4q31–35; 9q34; 6q23–q27; 2p22.1; and 9p12–p21.1. There was no strong correlation between the level of cellular maturation (as determined by monoclonal antibodies) and these six chromosome regions. The abnormality at bands p12 through p21.1 on chromosome 9 was found in two mature T cell lines (Leu 4+) but not in the other T cell lines (Leu 4−). Further studies comparing the karyotype and immunophenotype in malignant T cells are needed to determine the incidence and significance of these associations.

Table 5. Common Band(s) Involved in the Chromosome Rearrangements of Five T Cell Lines

SUP-T1	T-LL	14q11.2	4q31-q35	9q34	6q23-q27	2p22.1*	
SUP-T4	T-LL						9p12-p21.1†
SUP-T3	T-ALL						
SUP-T2	T-ALL						
K-T1	T-ALL						

*Both cell lines have rearrangements involving proximal bands on chromosome 2, band p22.1 (K-T1) and band p21 (SUP-T1).

†Both cell lines lacked chromosome 9 region p12→p21.1.

Both T-LL cell lines possessed a rearrangement involving chromosome 14 band q11.2. The inversion in chromosome 14, inv(14) (q11.2q32.3), appears specific for neoplastic T lymphocytes and has been found in T-CLL, T-cell lymphoma, and a lymphosarcoma cell line.[9,20,27] In addition, reciprocal translocations between the region of q11.2 of chromosome 14 and chromosomes 10, 11, and 14 have been observed in T-ALL, T-LL, and T-cell malignancies from patients with ataxia telangiectasia.[13,22] These observations led investigators to suspect that this chromosome band may be adjacent to genes that control cellular proliferation in general or ones that control specific aspects of T cell growth. Subsequent investigations determined that the gene for the α chain of the T cell receptor is located on chromosome 14 band 11.2.[23,24] Thus, T cell chromosomal translocations may be analogous to B cell malignancies that possess translocations involving the immunoglobulin heavy (or light) chain locus and the *myc* oncogene.[23,25]

The functional significance of the other chromosomal abnormalities that occurred in more than one T cell line are less well defined (Table 5). Two cell lines had a deletion of the long arm of chromosome 4 with a breakpoint at band 4q31. This breakpoint is adjacent to the gene for the T cell growth factor (interleukin 2, IL-2) which is reported to be located at band q26 through q28 on chromosome 4.[26] The cell lines have been established and maintained without supplemental IL-2, but T cell lines may produce IL-2.[27] In T lymphocytes which possess IL-2 receptors, IL-2 gene activation may be associated with excessive proliferation and/or neoplasia by an autocrine loop mechanism.[28]

Abnormalities involving chromosome 9 were observed in four of the five T cell lines. Two cell lines had an irregularity involving the distal end of the long arm of chromosome 9, whereas two other cell lines had an interstitial deletion of the short arm (Tables 4 and 5). Structural rearrangement on chromosome 9 are seen in ~20% of the cases of T cell malignancies and in many of the breakpoints on the short-arm cluster near band 9p21.[10,29] Nonrandom loss of chromosome material at 9p21 was recently observed in the karyotype from six of eight children with ALL with lymphomatous features.[30] Moreover, loss of 9p− may have clinical significance because the subgroup of ALL patients with this deletion appear to have a grave prognosis.[31] Breakpoints on the long arm of chromosome 9 tend to occur near band q34, the site of the *abl* oncogene.[10,31-34] The *abl* oncogene has been shown to be expressed in CML, AML, T cell lines, and malignant cells from patients with T-ALL.[34-36]

One T-ALL cell line (SUP-T2) has a single chromosome abnormality, a deletion of the long arm of chromosome 6, del 6 (q21q27). The 6q- abnormality is a chromosomal change observed in hematopoietic malignancies; it has been commonly identified in cases of diffuse large cell lymphoma, Japanese adult T cell leukemia and Sézary syndrome.[11,32,37-39] In pediatric malignancies, the 6q- abnormality occurs in ~10% of cases of common ALL and T cell ALL.[40,41] In these reports, the breakpoints in the long arm of chromosome 6 varied from band q15 to band q25 but tended to concentrate at band 6q21. The *myb* oncogene has been localized to chromosome 6 band q22-24, and expression of this oncogene routinely occurs in hematopoietic malignancies.[35,42] Another oncogene, n-*myc*, may also be expressed in T cell malignancies. Two cell lines had rearrangements involving proximal bands on chromosome 2 that were adjacent to or involved the site of the *n-myc* oncogene, which has been localized to chromosome 2 band p22p24.[43]

Table 6. Association of Specific Chromosomal Abnormalities in the Cell Lines With Sites of Known Oncogenes

Karyotype Analysis on T cell Lines			Oncogene[23,34,50]	
Cell Line	Cell Type	Specific Chromosome Abnormality	Type	Location
K-T1	T-ALL	t(1;11)(p36;p13)	*Src*	1(p34-p36)
		del(2)(p16p22)	*Myc*^{N}	2(p22-p24)
		t(1;11)(p36;p13)	*Ras*$^{H-1}$	11(p13-p15)
SUP-T2	T-ALL	del(6)(q21q27)	*Myb*	6(q22-q27)
SUP-T3	T-ALL	del(4)(q31q35)	*Raf*-2	4(p-q)
		del(9)(q32q34)	*Abl*	9(q34)
SUP-T1	T-LL	inv(2)(q11p21)	*Myc*^{N}	2(p22-p24)
		del(4)(q31q35)	*Raf*-2	4(p-q)
		del(6)(q23q27)	*Myb*	6(q22-q27)
		t(9;?)(q34;?)	*Abl*	9(q34)
		inv(14)(q11.1q32)	*tcl*-1*	14(q32.3)
SUP-T4	T-LL	t(1;20)(p22;q13)	*Src*	20(q12-q13)

*Proposed location of T cell lymphoma/leukemia-1 oncogene.[23]

Each of the five T cell lines had at least one chromosome breakpoint that involved a band reportedly possessing a proto-oncogene (Table 6).[34,44] Of the six chromosomal abnormalities seen in more than one T cell line, three are close to an oncogene site, and oncogene expression secondary to chromosomal breaks may be involved in the genesis of T cell neoplasia. An apparent inverse relationship exists in the cell lines between the number of oncogene sites involved and the length of the doubling time of the cell lines in vitro.

Chromosome weak points may be unmasked by procedures that stress cells growing in suspension cultures. These chromosomal fragile sites may readily break under the biologic stress of growth media containing: (a) low folic acid, (b) aphidicolin, or (3) caffeine.[45-47] Of the 21 chromosome breakpoints in the five T cell lines, 11 were located at or near fragile sites (Table 7). Chromosome changes at fragile sites may have occurred in the malignant cells in vivo (prior to

Table 7. Association of Specific Chromosomal Abnormalities in the T Cell Lines With Chromosome Fragile Sites

Constitutive Fragile Sites		Cell Line Abnormality	
Group	Chromosomal Locations	Cell Line	Chromosomal Changes
Caffeine-inducible	1p21.2	SUP-T4	t(1;20)(p22;q13)
	2p16.2	K-T1	del(2)(p22.1p16.3)
	4q31.1	SUP-T1	del(4)(q31q35)
		SUP-T3	del(4)(q31q35)
	7q32.3	SUP-T1	del(7)(q32q36)
Folate-sensitive	2q11	SUP-T1	inv(2)(p21q11)
	9p21	K-T1	del(9)(p12p21)
	10q23	SUP-T4	t(10;14)(q23;q11)
Aphidicolin-inducible	1p36	K-T1	t(1;11)(p36;p13)
	9q32	SUP-T3	del(9)(q32q34)
	11p13	K-T1	t(1;11)(p36;p13)

Table 8. Comparison of the Specific Chromosomal Abnormalities in the T Cell Lines with the Proposed Common Cancer Chromosome Breakpoints

Cell Line	Cell Type	Cell Line Chromosomal Abnormality	Common Cancer Chromosome Breakpoints
K-T1	T-ALL	t(1,11)(p36;p13)	1p36
		del(9)(p12p21)	9p21
SUP-T3	T-ALL	del(9)(q32q34)	9q34
SUP-T1	T-LL	del(6)(q23q26)	6q23
		t(9,?)(q34;?)	9q34
		inv(14)(q11 2q32)	14q32
SUP-T4	T-LL	t(9,16)(q12;q13)	9q13
		t(10;14)(q23,q11 2)	10q23

Derived from data in ref 48.

culture) or they may be secondary to the stress of proliferation in vitro. In the T cell lines reported, six specific chromosomal abnormalities occurred in more than one cell line. The fact that only one of these abnormalities (4q31) occurred at a fragile site supports the hypothesis that the fragile site changes occurred in vivo.

Structural karyotypic aberrations in human malignancies may be primary changes associated with cancer cell growth or may be secondary to excessive cellular proliferation alone. Recently, Mitelman reported a restricted number of chromosomal regions may be implicated in the etiology of human malignancies.[48] By using a registry of malignancies karyotyped with banding studies, he found 54 common cancer chromosome abnormalities, which were present in >90% of 3,844 registry cases.[49] The 54 reported common cancer chromosome abnormalities were restricted to 61 bands. Of the 21 chromosome breakpoints in the five T cell lines presented, seven were located at or adjacent to sites of common cancer chromosome changes (Table 8).

Thus, many of the karyotype changes in the T cell lines are associated with chromosomal abnormalities commonly observed in cancer. In addition, chromosome breakpoints are close to oncogenes or genes that may control specific aspects of T cell growth (IL-2 α chain of the T cell receptor). Because cell lines can proliferate indefinitely, they may provide the basic material for investigations defining the biologic significance of specific chromosome changes.

NOTE ADDED IN PROOF

Recently, a cell line (SUP-T5) was established from a patient with T-LL which possessed a unique translocation between chromosome 7 and chromosome 9: t(7;9)(q34;q34.3).[51] Upon re-examination of the karyotypes of the cell lines reported here (with specific attention to chromosomes 7 and 9), a t(7;9)(q34;q34 3) was found in the SUP-T1 cell line while a t(7,9)(q34;q32) was found in the SUP-T3 cell line Thus, band q34 on chromosome 7 may be an additional common chromosome breakpoint in T cell malignancies.

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Spontaneous Tumor Cytolysis Mediated by Inflammatory Neutrophils: Dependence Upon Divalent Cations and Reduced Oxygen Intermediates

By Alan Lichtenstein

The role of divalent cations and reactive products of the respiratory burst were investigated in spontaneous tumor lysis mediated by inflammatory neutrophils (PMNs). Murine peritoneal PMNs, obtained five hours after intraperitoneal injection of bacteria, conjugated and lysed teratocarcinoma cells in chromium release and single-cell cytotoxicity assays. The presence of extracellular magnesium was required and was sufficient for tumor cell binding to PMNs. Postbinding lytic events depended upon the simultaneous presence of extracellular calcium and magnesium. Catalase and superoxide dismutase inhibited postbinding lytic events, indicating that production of reduced oxygen moieties was important. Scavengers of hydroxyl radicals could inhibit tumor cell binding, but none could affect postbinding lytic events. Neither could inhibitors of myeloperoxidase decrease tumor lysis. The ability of conjugating PMNs to lyse their bound targets correlated with their reduction of nitro blue tetrazolium (NBT). Optimal concentrations of phorbol myristate acetate (PMA) markedly increased the NBT positivity of PMNs and the killing of bound tumor cells. Even with optimal stimulation of the respiratory burst, however, there was still a significant number (19%) of bound targets that escaped lysis, suggesting active resistance to oxygen-mediated tumor cell injury.

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PREVIOUS STUDIES have demonstrated that neutrophils (PMNs) must be activated in vitro before they can exert significant extracellular tumor cytotoxicity.[1-5] Almost all of these studies, however, have assayed peripheral blood (PB) PMNs; inflammatory PMNs may be better suited for tumor cytolysis. Our previous studies[6-8] support this hypothesis. Peritoneal PMNs, elicited as part of an acute inflammatory response to injection of killed bacteria or thioglycolate, exerted significant and rapid lysis of a variety of murine or human tumor cells [6,7] Simple binding to tumor cells was not sufficient because lysis also depended on temperature-dependent postbinding events.[8] Lysis was initiated rapidly and completed within 30 to 45 minutes after membrane contact with tumor cells.[6,7]

The activation of PMNs as they migrate into sites of inflammation may explain their acquired ability to exert spontaneous tumor lysis. In particular, exposure to chemotactic peptides[4,9] or leukotriene B_4[10] markedly enhances the ability of PMNs to undergo a respiratory burst upon subsequent activation, with the resulting production of huge quantities of reactive oxygen intermediates (ROIs). As previously demonstrated,[11,12] these reduced oxygen species are capable of inducing significant tumor cytolysis. Thus, we initiated these studies to assess the role of oxygen metabolites in spontaneous tumor lysis mediated by inflammatory PMNs. The results confirm that lysis is due to the concomitant production of hydrogen peroxide (H_2O_2) and superoxide (SO).

In addition to a chromium release assay (CRA), we used a cytotoxicity assay, which enables visualization of effector–target interactions at the single cell level[8] and allows a separation of PMN tumor lysis into two distinct phases: tumor cell binding and postbinding lytic events. A previous report detailed the disparate temperature requirements for each phase.[8] The current study further supports the hypothesis that the two phases are under separate regulatory control: magnesium ions were required for tumor cell binding whereas calcium as well as magnesium ions were required for postbinding lytic events.

MATERIALS AND METHODS

Mice Female C3HeB/FeJ mice, 6 to 8 weeks of age, were obtained from the Jackson Laboratory (Bar Harbor, Me) and were rested in our vivarium for one week before initiation of an experiment.

Tumor. The murine ovarian teratocarcinoma (MOT)[13] is maintained by weekly intraperitoneal (IP) passage in C3HeB/FeJ mice.

Drugs. *Corynebacterium parvum (C parvum,* strain CN6134) was purchased from Burroughs Wellcome (Research Triangle Park, NC). Thioglycolate broth was obtained from Difco (Detroit, Mich) and was injected IP as a 10% solution The following drugs were purchased from SIGMA (St Louis): EDTA, EGTA, agarose, nitro blue tetrazolium (NBT), superoxide dismutase (SOD), catalase, phorbol myristate acetate (PMA), sodium benzoate, D-mannitol, potassium cyanide, L-tryptophan, thiourea, ethylene glycol, L-histidine, methimazole, and aminotriazole. Dimethyl sulfoxide (DMSO), calcium chloride, and magnesium chloride were obtained from Mallinckrodt (St Louis), and sodium azide was obtained from J.T. Baker Chemicals (Phillipsburg, NJ) PMA was stored in ethanol at −20 °C. Several lots of catalase and SOD were tested until we identified reagents that lacked contamination by the other enzyme.

Treatment and lavage of mice. Mice were injected IP with a 0 2-mL solution containing 1,400 μg of *C parvum* or 0 4 mL of a 10% solution of thioglycolate. Five hours after being injected, the mice were killed Their peritoneal cavities were lavaged by rapid injection of 10 mL of cold phosphate-buffered saline (PBS) Peritoneal fluid was withdrawn and reinjected twice more. After washing, peritoneal exudate cells (PECs) were counted, resuspended in media, and used directly in chromium release assays, or were fractionated on Percoll gradients in preparation for conjugate and single-cell cytotoxicity assays. Viability of PECs after lavage was always >97%

CRA. Targets were radiolabeled by incubating 1×10^7 MOT cells with 50 μCi of ^{51}Cr for one hour at 37 °C in a shaking water bath in RPMI medium supplemented with 10% fetal calf serum

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(FCS, Reheis, Ariz) PECs (0 1 mL) were mixed with 1 × 10^4 radiolabeled target cells (0.1 mL) at different effector–target (ET) cell ratios in microtiter plates. Samples were run in quadruplicate. After four hours of incubation at 37 °C in a 5% CO_2 atmosphere, plates were centrifuged (1,200 rpm for ten minutes), and 0.1 mL of supernatant was removed and counted in a gamma counter Percentage of specific lysis was determined by: cpm experimental group − cpm minimal release/cpm maximal release − cpm minimal release × 100 Minimal release was determined by incubating targets in medium alone for four hours. Maximal release was determined by adding HCl to a concentration of ~1 mol/L. Data are presented as percentage of specific release at different ET ratios, mean ± SD of three to four separate experiments We have previously documented that significant in vitro lysis of MOT targets, mediated by PECs obtained 12 hours after IP injection of *C parvum* or thioglycolate, is due to the action of PMNs [6,7]

Percoll gradient fractionation of PECs Percoll fractionation of PECs (obtained five hours after IP injection) was performed as previously described.[6,7] In brief, a self-generating Percoll gradient was formed by mixing 1 mL of PECs (1.5 × 10^7/mL in RPMI and 10% FCS) with 8 mL of 70% Percoll (Pharmacia, Piscataway, NJ) in 10-mL high-speed centrifuge tubes. Tubes were spun at 60,000 g for 20 minutes at 15 °C in a Beckman high-speed centrifuge with a Ti-50 fixed angle rotor The PMN-enriched, macrophage-depleted cell fraction was found in a band corresponding to a density range of 1 075 to 1.090. Viability was always >94%. PMN-enriched fractions contained 92% to 97% PMNs, 3% to 8% lymphocytes, and 0% to 1% macrophages

Single-cell cytotoxicity assay (SCCA) The SCAA was performed as a modification of the method of Grimm and Bonavida[14] as previously described [6–8] In brief, PECs were first fractionated on the self-generating Percoll gradient and were then washed and resuspended to 2 × 10^6/mL in RPMI and 1% FCS. MOT targets were washed and resuspended to 2 to 10 × 10^6/mL (depending upon desired ET ratio) in RPMI and 1% FCS All experiments depicted were performed at ET ratio of 1:5 unless otherwise indicated. Targets and effectors were kept separately at 30 °C for five minutes prior to conjugation. Effectors (0.15 mL) were then mixed with targets (0 15 mL) in 10 × 75 borosilicate tubes and incubated at 30 °C for ten minutes. The tubes were centrifuged at 200 g for five minutes, and the supernatant was decanted. After 0.05 mL of RPMI and 1% FCS was added, the cell pellet was gently resuspended by slowly drawing the mixture into a pipette tip (50-μl MLA pipette) and slowly expelling it back into the tube Molten agarose at 39 °C was quickly added (50 μL) to each tube and mixed by brief rotation. The agarose–cell mixture was then spread onto agarose-coated slides. The slides were then submerged in RPMI and 1% FCS for incubation at 37 °C in a 5% CO_2 incubator. After one hour, slides were immersed in 0.2% trypan blue for five minutes to stain dead cells and were rinsed in 0 3% formalin until excess stain was removed. Slides were kept in 0.3% formalin until examination

To identify effector cells, trypan-stained slides were flooded with safranin for 90 seconds and then immersed in water for 60 seconds to remove excess stain When examined under oil immersion, living cells stain orange and dead cells stain purple. Percentage of conjugation was calculated as follows number of conjugates/total number of PMNs (bound and unbound) × 100 Percentage of killing was calculated as follows: number of dead targets in conjugates/total number of conjugates × 100. At least 200 PMNs/slide were examined and enumerated to determine percentage of conjugation and percentage of killing The slides were run in triplicate, and the standard deviation (SD) was always <5% of the mean values. The data is presented as mean ± SE of three to four separate experiments.

We have previously demonstrated[6–8] that all conjugates consist of either one target bound to one PMN (90%) or one target bound to two or more PMNs (10%). Control slides containing targets alone consistently demonstrated >96% viability during a one-hour incubation In addition, unbound targets as well as bound and unbound PMNs consistently demonstrated >95% viability during the assay Thus, on experimental slides, loss of viability (trypan positivity) occurred only in the bound target cell population.

Cytotoxicity assays with enzyme inhibitors and scavengers. PMNs and targets were individually suspended in media containing different concentrations of inhibitors or scavengers and were kept in these solutions for five minutes at 30 °C. In the CRA, PMNs and targets were then mixed in microtiter plates, centrifuged, and incubated for four hours In the SCCA, PMNs and targets were mixed for ten minutes, centrifuged at 200 g for five minutes, the supernatants were decanted, and new media and agarose containing the same concentrations of inhibitors or scavengers were added. Thus, PMNs were in contact with inhibitors/scavengers before conjugation and remained in contact until termination of the incubation. In separate control slides, exposure of tumor cells alone (no PMNs) to these concentrations of inhibitors/scavengers did not affect viability In CRAs, the spontaneous release of radioisotope from targets was not affected by these concentrations of inhibitors/scavengers.

Pretreatment with cyanide PMNs and MOT targets were suspended at 2 × 10^6/mL in RPMI media, 1% FCS, and varying concentrations of potassium cyanide (at pH = 7.4) for incubation at 4 °C After 30 minutes, the cells were washed four times, counted, and resuspended for cytotoxicity assays. Viability after cyanide pretreatment was always >95%.

Calcium pulse experiments The calcium pulse technique was used as previously described.[15] In brief, PMNs and tumor targets were mixed in the presence of EGTA (1 mmol/L), which allowed conjugation to occur normally but prevented initiation of lysis by chelating calcium Calcium chloride (10 mmol/L) was then added to initiate lysis in the presence or absence of the scavenger in question Incubation was continued at 37 °C for one hour before examination of PMN-target conjugates for determination of percentage of binding and percentage of killing. Control slides on which cells were conjugated and resuspended in EGTA *without* calcium supplementation were examined to ensure that the chelation of calcium prevented tumor cytolysis

Use of NBT in SCCAs The reduction of NBT to dark blue formazan deposits was used to detect PMNs actively producing reduced oxygen moieties. After PMNs and targets were mixed and centrifuged, the supernatant was decanted Fifty lambda of 0.1% NBT (in RPMI) was added to the cell pellet. An equal volume of molten agarose was then added before the cell mixtures were plated on slides to bring the final concentration of NBT to 0.05%. Slides were then immersed in media containing 0.05% NBT for the one-hour incubation at 37 °C All slides containing NBT were kept protected from light until examination. Staining with trypan and safranin after completion of incubation was performed as described above

Determination of optimum concentration of PMA for PMN stimulation Peritoneal PMNs were obtained five hours after IP injection of *C parvum* and were stimulated with PMA. Hydrogen peroxide (H_2O_2) production was measured by the loss of fluorescence from scopoletin [16] Hydrogen peroxide produced by PMA-stimulated PMNs was plotted against a standard curve of scopoletin reduction, which was generated by sequential additions of 1 nmol of reagent grade H_2O_2 in the presence of 10^{-4} mol/L of scopoletin (SIGMA) and 1 μg/mL of horseradish peroxidase (SIGMA) Under these conditions, 10^{-7} mol/L of PMA stimulated the greatest release of H_2O_2 (9 2 nmol/L of H_2O_2/10^6 PMNs per five minutes).

Stimulation with PMA in SCCA. After PMNs and targets were mixed and centrifuged, the supernatant was decanted. Fifty lambda

Table 1. Inflammatory PMNs Lyse Tumor Targets In Vitro

Chromium Release Assay*					
				Specific Release‡	
	Cells (%)†			E–T	
Effectors	PMNs	Lymphocytes	Macrophages	40.1	20.1
Unfractionated PECs	93 ± 4	4 ± 1	3 ± 1	30 ± 3.9	15 ± 2.1
Fraction 4	97 ± 3	3 ± 1	0	32 ± 3.2	17 ± 2 6
Fraction 1	22 ± 5	4 ± 1	74 ± 4	3 ± 0.4	− 1 ± 0 9

Single-Cell Cytotoxicity Assay Effectors	Effectors (%) Bound§ to MOT Targets (x̄ ± SD)	Conjugated Targets (%)§ Lysed After 3-h Incubation (x̄ ± SD)
Neutrophils	18 ± 2.5	65 ± 4
Lymphocytes	12.5 ± 3.1	0

*PECs were obtained five hours following IP injection of 1,400 μg of formalin-killed bacteria. PECs were then fractionated on self-generating Percoll gradients resulting in a PMN-enriched fraction (fraction 4) and a PMN-depleted fraction (fraction 1). PECs from both fractions were used as effectors in a 4-h chromium release assay against MOT targets. In addition, PECs of fraction 4 (PMN-enriched) were used as effectors in the single-cell cytotoxicity assay.

†Percentage of cells, x̄ ± SD of four experiments as determined on Wright's stained smears

‡Percentage of specific lysis, x̄ ± SD of four experiments at effector–target (E–T) ratios of 40 and 20:1

§Percentage of binding and percentage of killing of either PMNs or lymphocytes, x̄ ± SE of four experiments; E–T, 1 5

PMNs, neutrophils; PECs, peritoneal exudate cells, MOT, murine ovarian teratocarcinoma.

of PMA (2×10^{-7} mol/L) was added to the cell pellet and an equal volume of molten agarose was added to give a final concentration of 10^{-7} mol/L PMA (10^{-7} mol/L) was also present during the one-hour incubation

Statistics The *t* test was used for all *P* value determinations.

RESULTS

Inflammatory neutrophils spontaneously lyse tumor targets. PECs, obtained five hours following IP injection of formalin-killed bacteria contain 93% ± 4% PMNs, 4% ± 1% lymphocytes, and 3% ± 1% macrophages (mean ± SD for four experiments). Tumor lysis mediated by these PECs is due to the effects of PMNs (Table 1). First, PECs enriched for PMNs and depleted of macrophages (fraction 4 PECs) are effective, whereas cell populations depleted of PMNs (fraction 1 PECs) are ineffective in exerting cytolysis. Second, only peritoneal PMNs were capable of binding and lysing targets in the SCCA. Although lymphocytes could bind, they could not lyse targets during the incubation period. Third, pretreatment with anti-theta or anti-asialo-GM$_1$ antibodies and complement, which effectively depleted PECs of T lymphocyte [responsiveness to phytohemagglutinin (PHA) and Concanavilin A (Con A)] and NK lymphocyte (lysis of YAC-1 targets) activity, did not affect the lysis of MOT targets (data not shown). These data are consistent with our earlier studies[6–8] documenting spontaneous tumor lysis by inflammatory PMNs.

The role of divalent cations in PMN-mediated tumor cytolysis. Initial experiments using the CRA demonstrated that the addition of EDTA (1 mmol/L) or EGTA (1 mmol/L) significantly suppressed PMN-mediated cytolysis (Table 2). We then used the SCCA, which enabled an independent evaluation of tumor cell binding and postbinding lytic events. When effector PMNs and targets were mixed in 1 mmol/L of EDTA, but not EGTA, significant inhibition of tumor cell conjugation occurred (Table 2) Because the former chelates calcium and magnesium and the latter chelates only calcium, these data indicated a requirement for magnesium ions in the tumor-binding phase of lysis. Table 2 also demonstrates that inhibition of binding was

Table 2. Divalent Cation Requirements for PMN Binding to Tumor Cells

Supplements Added to Assay	(%) Specific Release*		Conjugation (%)†	
	E–T			
	40 1	20·1	Exp. 1	Exp. 2
A. 0 (control group)	25 ± 2	12 ± 1	17.4 ± 2.5	15.8 ± 3.2
B EDTA (1 mmol)	3 ± 1‡	1 ± 0.4‡	2 1 ± 0 7‡	3.1 ± 0.6‡
C. EGTA (1 mmol/L)	4 ± 1‡	1 ± 0.2‡	16 6 ± 2.0	15.2 ± 1.9
D EDTA (1 mmol/L) + magnesium (10 mmol/L)		—	18.2 ± 3.1	16 2 ± 3.6
E. EDTA (1 mmol/L) + calcium (10 mmol/L)		—	2.6 ± 0.5‡	2 9 ± 1.0‡

Peritoneal cells were obtained five hours after intraperitoneal injection of *C parvum* and mixed with murine ovarian teratocarcinoma targets Supplements were added to media used in conjugation assay.

*Data are presented as percentage of specific release, mean ± SD of three separate experiments.

†Data are presented as percentage of conjugation, mean ± SD of triplicate slides for each experiment, effector–target (ET) ratio 1:5. EDTA also inhibited conjugate formation at ET1:1 and 1:10 (not shown).

‡Significantly lower than control (group A), $P < .05$

Table 3. Requirement for Calcium in Postbinding Lytic Events

Group	Exp 1* Conjugation (%)	Exp 1* Killing (%)	Exp 2 Conjugation (%)	Exp 2 Killing (%)
A Control	14.5 ± 1.7	40 ± 4	17.7 ± 2.1	39 ± 4
B. Bind in EGTA (1 mmol/L) and incubate in EGTA (1 mmol/L)	12.9 ± 2.1	2 ± 0.6†	16.8 ± 3.0	6 ± 1†
C Bind in EGTA (1 mmol/L and incubate in EGTA with excess calcium (10 mmol/L)	13.0 ± 1.6	42 ± 3	16.3 ± 2.2	36 ± 3

Peritoneal cells obtained five hours after IP injection of *C parvum* and mixed with MOT targets, E.T=1·5. Supplements added as described

*Data presented as percentage of conjugation and percentage killing, mean ± SD of triplicate slides for each experiment.

†Significantly lower than control group (group A), *P* < .05

reversed by the addition of 10 mmol/L of exogenous magnesium, indicating that EDTA did not cause an irreversible nonspecific suppression of PMN function. Inhibition of binding by EDTA could not be reversed by exogenous calcium (Table 2), further indicating calcium is not required for binding.

Because EGTA did not affect the binding step but significantly suppressed chromium release (Table 2), the data suggested that calcium was required for postbinding lytic events. To test this hypothesis, PMNs and targets were first mixed for ten minutes in EGTA. Because free magnesium was present, binding to tumor cells occurred normally (Table 3). After centrifugation, conjugates were resuspended in agarose-media with the identical concentration of EGTA present. The percentage of killing was significantly suppressed in this group (line B, Table 3) indicating a requirement for calcium during the postbinding lytic events. When these events occurred in EGTA with excess calcium added (line C), the percentage of killing was comparable to that of the control group (line A), indicating that EGTA did not irreversibly affect PMNs to prevent them from lysing targets. Tumor cells incubated alone (no PMN effectors) in EGTA (1 mmol/L) with or without excess calcium (10 mmol/L) maintained viability >93%

To assess the role of magnesium during postbinding lytic events, PMNs and tumor cells were mixed in EGTA, which allowed normal binding but prevented lysis from occurring (by chelating calcium). After centrifugation, conjugates were resuspended and incubated in agarose-media to which different supplements were added (Table 4). In line A, excess calcium was added and significant lysis occurred. In line B, the incubation was performed in EDTA and lysis was completely inhibited, indicating that calcium or magnesium or both are required for postbinding lytic events. Lines C, D, and E in Table 4 demonstrate that both cations are required for the lytic events because only when both are replaced in the incubation media is the percentage of killing normalized. Control slides of tumor cells alone (no effectors) incubated in EDTA (1 mmol/L) with calcium (10 mmol/L) or magnesium (10 mmol/L) demonstrated viability >94%.

Tumor lysis depends upon hydrogen peroxide and superoxide production by PMNs. We next investigated the role of oxygen metabolites by adding enzyme inhibitors of ROIs to the cytotoxicity assays. Addition of catalase or SOD inhibited tumor lysis in a concentration-dependent fashion (Table 5). There was no inhibition of tumor cell binding, but postbinding lytic events were significantly inhibited. Furthermore, heat-inactivated enzymes were not inhibitory, ruling out a nonspecific toxic effect on PMNs. Tumor cells incubated alone (no effectors) with catalase and/or SOD maintained a viability >92%. Inhibition of lysis achieved by adding both enzymes together was significantly greater than it was when either one was used separately (Table 5). This suggested that hydroxyl radicals, formed by a reaction between superoxide and H_2O_2 (Haber-Weiss reaction) were important mediators of tumor lysis. To test this hypothesis, several scavengers of hydroxyl radicals were added to CRA or single-cell cytotoxicity assays Histidine (0.25 to 25 mmol/L, tryptophane (0.25 to 25 mmol/L), and ethylene glycol (0.32 mol/L) had no effect on percentage of specific lysis, percentage of binding or percentage of killing of targets (data not shown). Thiourea suppressed cytotoxicity in the

Table 4. Requirement for Magnesium in Postbinding Lytic Events

Supplements Added During Incubation	Exp 1* Conjugation (%)	Exp 1* Killing (%)	Exp 2 Conjugation (%)	Exp 2 Killing (%)
A. calcium (10 mmol/L)	22.5 ± 2.1	47 ± 3	20.6 ± 1.5	53 ± 4
B EDTA (1 mmol/L)	20 ± 1.5	5 ± 1†	18.7 ± 1.7	8 ± 2†
C. EDTA (1 mmol/L) and calcium (10 mmol/L)	21 ± 1.8	8 ± 2†	19.5 ± 1.9	11 ± 2†
D EDTA (1 mmol/L) and magnesium (10 mmol/L)	19.5 ± 2.3	11 ± 3†	18.9 ± 2.0	10 ± 1†
E. EDTA (1 mmol/L), calcium (10 mmol/L) and magnesium (10 mmol/L)	24.3 ± 2.0	48 ± 4	20.1 ± 2.2	49 ± 5

PMNs obtained five hours after IP injection of *C parvum* and mixed with MOT cells (ET1:5) in EGTA (1 mmol/L) Supplements added during incubation step

*Data presented as percentage of conjugation and percentage of killing, mean ± SD of triplicate slides for each experiment

†Significantly decreased as compared to control (group A), *P* < .05

Table 5. Inhibition of PMN-Mediated Tumor Lysis by Catalase and SOD

Inhibitor Added	Specific Release (%)* ET 40 1	Specific Release (%)* ET 20·1	Binding (%)†	Killing (%)†
A. 0 (control)	21 ± 3	12 ± 3	10.6 ± 1 1	60 ± 5
B Catalase 500 U/mL	11 ± 1‡	5 ± 1 5‡	11 1 ± 1.6	28 ± 2 5‡
C Catalase 5,000 U/mL	6 ± 1‡	3 ± 0 3‡	12.2 ± 1.0	18 ± 1.7‡
D Catalase 5,000 U/mL, HI§	20 ± 2	12.2 ± 2.1	10 5 ± 1.3	66 ± 4.5
E SOD 125 U/mL	8 ± 1‡	2 ± 0 2‡	10.0 ± 0.7	33 ± 2.0‡
F SOD 250 U/mL	—	—	9 1 ± 1.1	20 ± 2.3‡
G SOD 250 U/mL HI§	22 ± 1.5	13 ± 1.1	9.8 ± 1.2	57 ± 3 1
H Catalase 5,000 U/mL + SOD 125 U/mL	—	—	11 1 ± 1.4	4 ± 1‡

PMN, neutrophil; SOD, superoxide dismutase; ET, effector–target ratio; MOT, murine ovarian teratocarcinoma.

C parvum-elicited PMNs mixed with MOT targets and incubated in the presence or absence of various concentrations of catalase or SOD.

*Percentage of specific release, mean ± SD of three separate experiments

†Percentage of binding and percentage of killing, mean ± SE of four separate experiments.

‡Significantly lower than control (group A), $P <$ 05.

§HI, heat inactivated, 100 °C for 15 min

CRA; however, this was due solely to an inhibition of tumor cell binding (Table 6). Killing of bound tumor cells was not affected by these concentrations of thiourea. Mannitol, DMSO, and benzoic acid all suppressed chromium release in a concentration-dependent fashion. These drugs markedly inhibited tumor cell binding by inflammatory PMNs so that their effects on postbinding lytic events could not be assessed. We therefore added the drugs to PMN–tumor cell mixtures that had successfully completed the conjugation step (Table 7, calcium pulse technique). Neutrophils and targets were conjugated in EGTA (line C, Table 7), which allowed normal tumor cell binding but prevented lysis. Conjugates were then incubated in excess calcium with (line E) or without (line D) the inhibitory concentrations of the scavenger in question. As shown in Table 7, these drugs have no effect on tumor lysis when added to cell mixtures that have successfully completed the binding step. As a positive control for suppression, we added catalase (500 U/mL) to cell mixtures that had completed the binding step in the calcium pulse technique and detected a decrease in killing from 55% to 21%.

Effects of myeloperoxidase inhibitors. To assess whether H_2O_2 induced tumor lysis by first interacting with myeloperoxidase and halide ions, several peroxidase inhibitors were added to the cytotoxicity assays. Aminotriazole, methimazole, cyanide, or azide had no effect on percentage of specific lysis, percentage of conjugation, or percentage of killing in the SCCA (Table 8). Because azide and cyanide prevent oxidative phosphorylation and might inhibit any tumor cell catalase present, they might also increase the sensitivity of targets to lysis, which could counteract any inhibitory effect on production of a cytotoxin by PMNs. Therefore, we performed experiments in which PMNs as well as targets were pretreated with cyanide (pH 7.4) and then washed extensively before they were mixed in the conjugation step. As shown in Table 8, pretreatment had no effect on PMNs but increased the susceptibility of targets to PMN-mediated lysis. These data support the notion that the

Table 6. Effect of Hydroxyl Radical Scavengers on PMN Cytotoxicity

Scavenger Added	Concentration (mmol/L)	Specific Release (%)* ET 40	Specific Release (%)* ET 20	Binding (%)†	Killing (%)†
0 (Control)	—	23 ± 2 5	14 ± 1 3	13 ± 1	84 ± 6
Thiourea	50	12 ± 1 1‡	6 ± 0.5‡	5 ± 0.2‡	79 ± 5
	10	14 ± 1.7‡	11 ± 2.7	8 ± 1 2‡	80 ± 3
DMSO	750	3 ± 0.8‡	1 ± 0.3‡	2 ± 0.2‡	—
	150	8 ± 1.1‡	1 ± 0.4‡	3 ± 0.3‡	—
Mannitol	50	4.2 ± 1.2‡	3 ± 0 5‡	3 ± 0.2‡	—
	5	20 ± 3 1	12 ± 0 8	11.6 ± 1.3	91 ± 4.1
Benzoic acid	50	3.0 ± 1.2	0 5 ± 0.2‡	2 5 ± 0.8‡	—
	5	19 ± 2.4	12 ± 1.6	10 ± 1.1	88 ± 3.5

PMNs, neutrophils, DMSO, dimethyl sulfoxide

C parvum-elicited PMNs mixed with murine ovarian teratocarcinoma targets in the presence or absence of various concentrations of hydroxyl radical scavengers.

*Percentage of specific release, mean ± SD of three separate experiments.

†Percentage of binding and percentage of killing, mean ± SE of three separate experiments

‡Significantly lower than control ($P <$ 05).

Table 7. Calcium Pulse Experiments

Group	DMSO 750 mmol/L		Mannitol 50 mmol/L		Benzoic Acid 50 mmol/L		Catalase, 500 U/mL	
	Binding (%)	Killing (%)	Binding (%)	Killing (%)	Binding (%)	Killing (%)	Binding (%)	Killing (%)
A PMN + tumor	14 ± 2	45 ± 4	10 1 ± 2 1	50 ± 4 5	10 4 ± 1 3	39 ± 5 0	11 ± 1 2	61 ± 2 2
B Scavenger present throughout assay	3 ± 1*	—	4 ± 1 0*	—	4 9 ± 0.6*	—	10 ± 2 1	28 ± 2 4*
C. PMN + tumor in EGTA	12 ± 1 7	6 ± 1 1*	9 1 ± 1 2	9 ± 2 2*	9 7 ± 2 3	4 ± 0 6*	—	—
D PMN + tumor conjugated in EGTA, incubated in calcium	11 ± 1 5	41 ± 2 8	9 9 ± 0 7	45 ± 6 1	11 5 ± 2 1	33 ± 3 0	12 ± 1 5	55 ± 3
E PMN + tumor conjugated in EGTA, incubated in calcium and scavenger	12 ± 1 0	38 ± 2.9	11 1 ± 0 9	59 ± 4 5	9 1 ± 1 7	35 ± 5 1	11 8 ± 1 2	21 ± 2*

C parvum-elicited PMNs mixed with murine ovarian teratocarcinoma cells (ET 1 5), in presence (group B) or absence (group A) of specific scavenger or in presence of EGTA, 1 mmol/L (groups C, D, and E) After conjugation for ten minutes and centrifugation, agarose-cell mixtures were plated on slides and incubated for one hour at 37 °C Conjugates incubated alone (group A), in presence of scavenger (group B), in presence of EGTA (group C), in presence of EGTA and excess calcium, 10 mmol/L (group D); or in presence of EGTA, calcium, and scavenger (group E) Data presented as percentage of binding and percentage of killing, mean ± SE of three experiments

*Significantly lower than control (group A), $P < .05$

activity of myeloperoxidase is not required for effective tumor lysis.

Augmentation of tumor lysis by addition of PMA. As shown in Tables 1 through 5, significant numbers of conjugated tumor targets always remain viable during the incubation. Some conjugated targets may have escaped lysis because their bound PMNs produced insufficient levels of ROIs. To gain support for this hypothesis, we stained PMN-tumor cell mixtures with NBT to identify PMNs actively producing ROIs. We then attempted to correlate NBT positivity with tumor cell killing. The percentage of unbound PMNs elicited by IP injection of *C parvum* that stained positively with NBT ranged from 24 to 58, with a mean of 33% (Table 9). When SOD (200 U/mL) was present during preparation of PMNs and subsequent staining, NBT positivity was markedly reduced from 33% to 4% (Table 9). Peritoneal PMNs positively stained by NBT demonstrated coarse dark granules of formazan usually present asymetrically to one side of the cell. In contrast, MOT tumor targets were never stained with NBT.

Conjugates with dead tumor targets contained PMNs that were almost always NBT positive (Table 9). In contrast, conjugates with viable tumor cells contained PMNs that were positively stained for NBT only 29% of the time (Table 9). Thus, the ability of conjugating PMNs to kill their bound targets roughly correlates with their frequency of positive NBT staining.

These data suggested that bound tumor cells may evade cytolysis if their conjugating PMNs are insufficiently stimulated for an effective respiratory burst. To address this issue further, we incorporated increasing concentrations of PMA into the binding and killing phases of the SCCA. As shown in Table 10, the percentage of PMNs that conjugated targets was comparable between concentrations of PMA of 10^{-9} to 10^{-5} mol/L. However, the percentage of killing was related to the amount of PMA incorporated into the assay. Although killing could not be evaluated at 10^{-5} mol/L because of increased background toxicity on targets from the PMA, killing clearly peaked at 10^{-7} and 10^{-6} mol/L concentrations. These concentrations of PMA resulted in the greatest

Table 8. Effect of Myeloperoxidase Inhibitors on PMN Tumor Cytolysis

Inhibitor	Concentration (mol/L)	Specific Release (%)* ET 40 1	Specific Release (%)* ET 20.1	Binding (%)*	Killing (%)*
0 (Control)	—	20 ± 1 5	13 ± 0.7	13.9 ± 1 5	51 ± 4 2
Methimazole	1	18 ± 2 1	15 ± 1.2	12.9 ± 1.2	49 ± 3.4
	0.1	19 ± 2.3	14 ± 1 9	13.5 ± 1 1	52 ± 4.5
Aminotriazole	30	18 ± 3.5	11 ± 1.2	13 5 ± 1 5	48 ± 2 9
	1	16 ± 4 1	13 ± 2.1	14.1 ± 2.2	55 ± 2.8
	0 1	20 ± 2.4	14 ± 1.6	11.9 ± 2 3	54 ± 3.5
Azide	1	22 ± 1.8	14 ± 2.5	12.3 ± 1 2	46 ± 2.7
Cyanide	1	20 ± 2.1	13 ± 1.2	13.4 ± 2 2	53 ± 3 5
	10	—	—	14 4 ± 2.2	62 ± 3 7
		Cyanide Pretreatment†			
		(PMNs)	(Targets)		
		–	–	13 5 ± 2 3	51.4 ± 4.2
		+	–	14 2 ± 1.2	47.5 ± 2.4
		–	+	15 0 ± 2.7	67.0 ± 3.1‡
		+	+	12.1 ± 2 1	60.1 ± 2.5

C parvum-elicited PMNs mixed with murine ovarian teratocarcinoma cells and incubated in presence or absence of various concentrations of myeloperoxidase inhibitors.

*Percentage of specific release, mean ± SD and percentage of binding and percentage of killing, mean ± SE of three experiments.

†PMNs or targets pretreated with cyanide, 1 mmol/L.

‡Significantly different from control (no pretreatment with cyanide), $P < .05$.

Table 9. NBT Positivity of Peritoneal PMNs

Cells Examined	SOD*	NBT (%), Mean ± SE (Range)†
Unbound PMNs	—	33 ± 6 (24–58)
Unbound PMNs	+	4 ± 1 (3–5)‡
PMNs conjugated to live targets	—	29.4 ± 4.4 (23–36)
PMNs conjugated to dead targets	—	93 ± 4.1 (88–96)§

NBT, nitro blue tetrazolium, PMNs, neutrophils, SOD, superoxide dismutase; MOT, murine ovarian teratocarcinoma

PMNs obtained five hours after IP injection of *Corynebacteria* (1,400 μg), mixed with MOT targets (E:T=1:5) and examined at single cell level after staining with NBT.

*At 200 U/mL

†Percentage of NBT-positive cells, mean ± SE of five separate experiments

‡Significantly decreased NBT staining as compared with control (unbound PMNs elicited by bacteria and studied without SOD) $P < .05$.

§Significantly increased NBT staining as compared with corresponding PMNs conjugated to live targets, $P < .05$.

augmentation of hydrogen peroxide production when added to *C parvum*-elicited PMNs (data not shown). These data indicate that a significant number of viable conjugated tumor cells can be lysed if their bound PMNs can be further stimulated to produce greater quantities of ROIs. However, 17% of bound tumor cells were still viable even with maximal PMA stimulation. As shown in Fig 1, this optimal concentration of PMA (10^{-7} mol/L) markedly increased the percentage of NBT positivity of free and conjugating PMNs. Specifically, 93% of conjugates with viable tumor cells contained NBT + PMNs, supporting the hypothesis that they were stimulated effectively by PMA but were still unable to cause lysis.

DISCUSSION

The ability of freshly obtained peritoneal inflammatory PMNs to lyse tumor targets spontaneously is novel because peripheral blood PMNs require prior in vitro activation to achieve lysis.[1-5] This paradox emphasizes the different functional capacities of PMNs obtained from different sites. Our results indicate that spontaneous tumor lysis was mediated by ROIs. Because in vitro-activated PB PMNs also induce cytolysis by production of ROIs,[1-5] the data suggest that the process of inflammation activates PMNs in vivo for an enhanced tumor-lysing oxidative burst.

SOD and catalase inhibited tumor cytolysis in a concentration-dependent fashion whereas heat-inactivated enzymes had no effect. In addition, suppression of lysis was greater when both inhibitory enzymes were used together, suggesting that hydroxyl radicals, formed by a reaction between superoxide and H_2O_2, were the lytic molecules. However, several hydroxyl radical scavengers, used in a wide range of concentrations, had no effect on postbinding lytic events. Sole use of a CRA would have led us to conclude erroneously that several scavengers could inhibit lysis. The SCCA demonstrated that these drugs had an inhibitory effect solely on tumor cell binding. The scavengers had no effect when they were added after binding had been completed. Nathan et al[17] also detected an inhibition of tumor cell binding to cytotoxic effectors caused by scavengers of hydroxyl radicals. These effects on binding are potential pitfalls in the interpretation of CRAs performed in the presence of these agents.

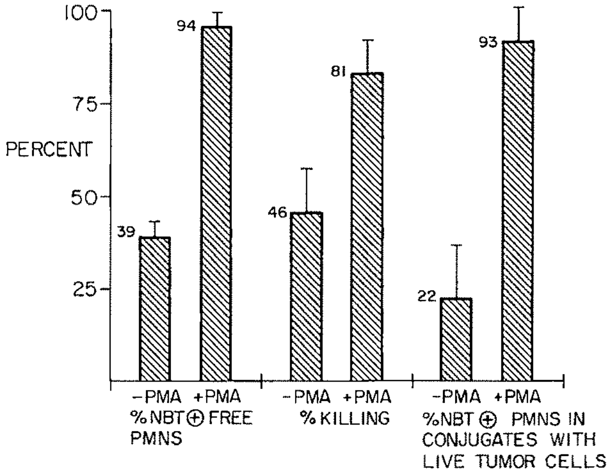

Fig 1. SCCA performed in the presence or absence of 10^{-7} mol/L of phorbol myristate acetate (PMA). Percentage of nitro blue tetrazolium (%NBT) positivity of free *C parvum*-elicited neutrophils (PMNs) was assessed on smears of peritoneal exudate cells (PECs) alone (no tumor targets), using triplicate slides per experiment. Data are presented as %NBT, mean ± SE of five separate experiments. Percentage of killing was determined with *C parvum*-elicited PMNs and murine ovarian teratocarcinoma (MOT) cells, effector–target ratio (E–T) 1:5, and is presented as percentage of killing (of bound tumor cells), mean ± SE of five separate experiments. Fifty-one conjugates with viable tumor cells were examined in the presence of PMA, and 82 conjugates were examined in the absence of PMA. Numbers next to bars are means.

It is possible that the scavengers tested could not gain access to the critical area of contact between PMN and tumor cell and therefore had no effect on postbinding lytic events. This may be a particular problem when the scavenger is added in the calcium pulse technique, ie, after binding is successfully completed. However, this is unlikely since protection against lysis was achieved by adding a large protein molecule such as catalase after binding was successfully completed.

A reaction between H_2O_2 and myeloperoxidase is not required for lytic effects to occur. Agents that inactivate myeloperoxidase had no effect on tumor lysis. Because azide and cyanide may affect targets by inactivating catalase and/or preventing oxidative phosphorylation, we pretreated PMNs and targets separately before initiating the cytotoxicity assay. Pretreatment of PMNs had no effect, but pretreatment of targets resulted in a modest increase in tumor cytolysis. Whether these latter effects are due to an inactivation of tumor cell catalase is unknown. However, it is clear that myeloperoxidase is not required for PMN-mediated lytic effects.

The lack of requirement for myeloperoxidase (MPO) is inconsistent with several previously reported studies.[1,2,12] However, studies by English and Lukens[4] and Nathan et al[18] demonstrated that MPO was not required for effective oxygen-mediated PMN lysis. One possible reason for these discrepancies is the different targets used in these studies. In experiments with the tumor target used in this study (MOT cells), the presence of halide and myeloperoxidase did not enhance lysis achieved by reagent grade H_2O_2 (unpublished observations).

The role of superoxide in PMN tumor cytotoxicity is not well established. Although human PMNs stimulated by lectins[19] or PMA[20,21] lyse erythrocytes through generation of superoxide, the only report to implicate superoxide in tumor cell killing is that of Mavier and Edgington,[22] who used PMN-stimulated peripheral blood monocytes as effectors. Although their SOD-induced protection from lysis was very modest, there appeared to be a synergistic cytotoxic interaction between superoxide and H_2O_2 (without evidence of a role for hydroxyl radicals), which is similar to results described here. Lysis of autologous erythrocytes by PMA-stimulated human PMNs also depends on the generation of superoxide and H_2O_2.[20] However, in this latter study, complete protection was afforded by either catalase or SOD (and not by hydroxyl radical scavengers), which implied an absolute requirement for simultaneous exposure to both oxygen metabolites.

The MOT tumor may be particularly sensitive to the lytic effects of superoxide as compared with previously studied targets. Further evidence of this sensitivity comes from preliminary experiments in which PMA stimulation of MOT-lysis by PMNs was significantly (but not completely) inhibited when SOD was present. In addition, PMA-stimulated lysis occurred equally under calcium-free conditions, thus correlating with the ability of PMA to stimulate superoxide release in the absence of calcium.

The additive effects of superoxide and H_2O_2 in the current study may be explained by a varying susceptibility to toxic oxygen molecules among the cells within our tumor population. Targets may be qualitatively heterogeneous, some primarily susceptible to superoxide-mediated damage and others susceptible only to H_2O_2. Thus, when catalase is added, H_2O_2-sensitive targets escape lysis; when SOD is added, superoxide-sensitive targets escape lysis. Alternatively, lysis may occur when a threshold of membrane damage is achieved by the additive effects of superoxide and H_2O_2. Tumor targets may then be quantitatively heterogenous in their individual threshold levels, susceptible targets being lysed by either H_2O_2 or superoxide alone and more resistant targets requiring the simultaneous presence of both metabolites for their effective lysis. Whichever theory is accurate, it is clear that there is variability among the targets in their susceptibility to lysis.

The activation of inflammatory PMNs in vivo that allows the production of ROIs in sufficient quantities for tumor lysis may be due to one or more of several stimuli. These cells have been exposed to chemotactic molecules and complement-opsonized bacteria during their egress into the peritoneal cavity and both are potent stimulators of the PMN respiratory burst.[23-25] It is also possible that these stimuli interact for an additive or synergistic release of oxygen metabolites. Pretreatment of leukocytes with chemotactic peptides[4,9] or opsonized zymosan[26] primes them for an increased respiratory burst following subsequent activation. It is quite possible, therefore, that in vivo exposures to chemoattractants and/or opsonized *Corynebacteria* effectively prime PMNs for a rapid and heightened production of superoxide and H_2O_2 when subsequent triggering occurs.

The presence of extracellular magnesium was necessary and sufficient for effective binding to tumor targets. These results are similar to those obtained when tumor cytotoxicity is mediated by sensitized T lymphocytes[27] or natural killer lymphocytes.[15] In contrast, only calcium is required for effective binding of antitumor macrophages to tumor targets.[28]

In contrast to binding, calcium was required for postbinding lytic events. Because these events involve the respiratory burst, calcium may be required for successful activation of PMN oxidative metabolism following binding to tumor cells. Calcium is needed for stimulation of the respiratory burst by many,[23,25,29-31] but not all[25] types of activators.

Magnesium may also be required for respiratory burst activation in some instances. Magnesium decreased the calcium requirements for superoxide release by digitonin-stimulated guinea pig PMNs.[29] An effective respiratory burst stimulated by serum-opsonized or C'3-opsonized zymosan also required the presence of magnesium as well as calcium.[25] In similar fashion, magnesium may be needed in addition to calcium for stimulation of tumoricidal PMNs. However, an alternative hypothesis is suggested by the strict requirement for magnesium in tumor cell binding. It is possible that when conjugates are resuspended in EDTA and agarose (Table 4), binding to tumor cells becomes sufficiently dissociated (even though this is not apparent microscopically) that effector cell stimulation does not occur. Thus, if only calcium is added back, PMNs are not sufficiently triggered by tumor cell contact to initiate the respiratory burst; if only magnesium is added back, membrane contact is sufficient but stimulation cannot proceed without extracellular calcium.

Most PMNs bound to viable tumor cells are NBT positive. Furthermore, the percentage of lysis could be significantly increased by incorporation of an optimal concentration PMA in the assay. Thus, a significant number of conjugating PMNs had not been activated for sufficient production of

Table 10. Enhancement of Tumor Lysis by Incorporation of PMA Into SCCA

Concentration of PMA (mol/L)	Conjugation (%)*	Killing (%)*
0	17.4 ± 1.5	48.7 ± 6
10^{-9}	17.7 ± 1.8	55 ± 7
10^{-8}	18.2 ± 2.2	62 ± 3†
10^{-7}	20 ± 2.0	83 ± 4†
10^{-6}	17.2 ± 2.2	77 ± 6†
10^{-5}	16.7 ± 1.8	—

PMA, phorbol myristate acetate; SCCA, single-cell cytotoxicity assay; MOT, murine ovarian teratocarcinoma.

C parvum-elicited peritoneal cells (five hours after IP injection) mixed with MOT targets, effector–target ratio 1:5, and increasing concentrations of PMA incorporated into the SCCA.

*Data presented as percentage of conjugation and percentage of killing, mean ± SD of triplicate slides. A second independent experiment gave identical results.

‡Percentage of killing significantly greater than control group (no PMA), $P < .05$.

oxygen metabolites necessary to effect tumor lysis; however, if further stimulated by PMA, they were capable of tumor cytotoxicity. It is unclear whether this is a random phenomenon or whether it represents true PMN heterogeneity, with specific subsets of cells being adequately stimulated for effective release of metabolites after tumor cell binding and other subsets requiring additional exogenous stimuli. The data in Table 10 and Fig 1 also indicate that some tumor cell resistance, at least in relative terms, exists in a subset of our targets. Even with optimal PMA stimulation, ~20% of bound tumor targets were still viable. Furthermore, most PMNs bound to these viable targets were stained positively with NBT, indicating that their production of reduced oxygen moieties had been significantly enhanced by PMA. Whether resistance is due to tumor cell catalase, as suggested by the data in Table 8, or to the operation of other biochemical pathways, is currently under investigation.

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Prognostically Significant Classification of Immune Changes in AIDS With Kaposi's Sarcoma

By Jeremy Taylor, Rahmat Afrasiabi, John L. Fahey, Elizabeth Korns, Michael Weaver, and Ronald Mitsuyasu

Sixteen immunological parameters were assessed quantitatively for their value in providing an immunologically-based and prognostically significant classification of the immune alteration in 97 patients with AIDS and Kaposi's sarcoma (AIDS-KS). The dimensions of reductions in the T4 (T helper-inducer cells) subpopulation of lymphoid cells in the T4–T8 ratio were found to correlate most closely with prognosis. Most other immunological changes did not relate to clinical course. T4 lymphocyte levels $>300/\mu L$ and a T4–T8 ratio >0.5 indicated a relatively good prognosis, eg, 85% to 95% survival at 12 months. T4 levels $<100/\mu L$ and/or a T4–T8 ratio <0.2 had a very poor prognosis, eg, $<25\%$ survival at 12 months. Intermediate T4 levels and T4–T8 ratios had intermediate prognosis. These immunological findings were found to have independent prognostic value for survival when compared with disease classifications based on tumor stage (I through IV) or on clinical status A (without) or B (with fever, night sweats, or weight loss). Reduced proliferative capacity, increased OKT10 antigen expression, elevated levels of serum IgA, and immune complexes also correlated with prognosis. Elevated levels of serum IgG, cellular HLA-DR expression, and skin test anergy occurred frequently in AIDS-KS but did not have prognostic significance. Variations in level of total lymphocyte, T8 (T suppressor/cytotoxic) cell, gamma FcR receptor-positive cell number, NK activity, or level of serum IgM were less common in AIDS-KS and did not correlate with prognosis.

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AIDS-ASSOCIATED Kaposi's sarcoma (AIDS-KS), in contrast to classical Kaposi's sarcoma, has an aggressive behavior.[1-4] Half of the patients have lymph node or gastrointestinal tract involvement at the time of diagnosis, and the disease generally has a progressive worsening clinical course over a period of months. AIDS-associated, epidemic, KS is associated characteristically with a profound suppression of cell-mediated immunity that is manifest as an absolute reduction in T4 (T helper/inducer) lymphocytes in the peripheral blood, resulting in an inversion of the T4–T8 lymphocyte subset ratio, reduced proliferative response to mitogens and alloantigens, and skin anergy to a variety of skin-test antigens.[3-6]

Because immunodeficiency is an essential element in the development of AIDS-KS and because patients with AIDS-KS differ in their course of disease, we attempted to determine which immune system changes, and which levels of change, were most relevant to prognosis. Ninety-seven patients with proven epidemic KS who were initially seen between January 1982 and February 1984 in the Kaposi's Sarcoma Clinic at UCLA Medical Center, were evaluated immunologically. Testing was performed on cellular and humoral immune parameters on presentation and at three-month intervals.

The relationship between immunologic parameters and clinical status (tumor stage, clinical subtypes A and B, and presence or absence of opportunistic infection) at the time of presentation and during the course of the disease was assessed. The prognostic value of the immunologic parameters at presentation on the course of the disease (survival and occurrence of opportunistic infection) was determined.

MATERIALS AND METHODS

Patients The study group consisted of 97 consecutive patients with biopsy-proven epidemic KS seen at the UCLA Kaposi's Sarcoma Clinic between January 1982 and February 1984 whose clinical and immunological status could be clearly determined Sixteen patients were excluded from the study because of inadequate histologic documentation of KS or because of incomplete clinical information Three patients with proven KS were excluded because their immunological status was within normal limits and because they showed no antibodies to HTLV-III. This group will be reported separately. All of the remaining patients from whom serum was available were found to have antibodies to HTLV-III virus. All patients were male homosexuals whose median age was 36 years with a range of 27 to 64 years There were no Haitians, Africans, or blood transfusion recipients among these patients. Patients were followed after their initial visit with clinical and immunological assessment at three-month intervals whenever possible.

In April 1985, 37 patients were still alive, 55 patients had died, and 5 were lost to follow-up. By June 1984, 56 patients had received treatment with α-interferon, 36 had received chemotherapy, and 3 were bone marrow transplant recipients, with many patients receiving more than one form of treatment; 25 patients had received no therapy. The results of these therapeutic trials have been reported[7-9] and are not considered in the scope of this study.

Fifteen patients had developed an opportunistic infection prior to, or at the time of initial presentation with KS (eg, *Pneumocystis carinii* pneumonia, *Candida* esophagitis, or *Toxoplasma gondii* encephalitis). Three patients had a history of lymphadenopathy prior to the diagnosis of KS. One patient had B cell non-Hodgkin's lymphoma diagnosed at the same time as KS.

The tumor stage (I through IV) and clinical subtypes A and B were established for each patient by a modification[4] of the criteria of Kriegel et al[10] on each date of immunological testing Tumor Stage I had limited cutaneous (one anatomic area) involvement and less than five cutaneous lesions; Stage II had disseminated cutaneous locations (more than one anatomic area) and more than five cutaneous lesions; Stage III had visceral involvement (gastrointestinal, lymph node, etc.); and Stage IV had both cutaneous and visceral

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involvement. Clinical subtypes were either. (A) asymptomatic; or (B) exhibiting fever of >100 °F, lasting more then two weeks and unrelated to an identifiable source of infection; night sweats; or weight loss of ≥10% of body weight

If no immunological testing had been done over an interval of more than three months but the patient had been seen clinically during that period, tumor stage and clinical subtypes were still noted Follow-up of patients who had left the Los Angeles area was accomplished by telephone interview whenever feasible. The median time of follow-up was 12 months, with a range of 1 to 33 months

Immunological testing Total T4 cell number, total T8 cell number, T4–T8 ratio, and HLA DR and OKT10 antigen expression were performed on peripheral blood of all patients at the time of their routine clinical assessments approximately every three months. Tests for phytohemagglutinin (PHA) proliferative response. natural killer (NK) activity, and FcR cell tests were usually done at initial presentation and then periodically on most patients Skin testing was conducted on 51 patients at their initial visit only. The serum immunoglobulins IgG, IgA, and IgM, and immune complexes were measured at the initial clinical evaluation and were not generally repeated

Peripheral blood mononuclear cells (PBMCs) were obtained as either heparinized (functional assays) or EDTA-treated (phenotype assays) venous blood. Total lymphocyte count and T cell subsets determination were accomplished by surface phenotype analysis using fluorescent monoclonal anti-T cell antibodies [6]

In brief, 50 μL of whole blood was treated with a RBC lysing reagent. The residual PBMCs were studied with stained fluorescein-conjugated monoclonal antibodies. T cell subpopulations were determined using an Ortho Spectrum III fluorescent-cell analyzer (Raritan, NJ) The monoclonal antibodies used and the respective mononuclear cell subpopulations are as follows· Leu4 (pan-T cell marker); Leu3a (T helper/inducer, T4); Leu2a (T suppressor/cytotoxic, T8) (Becton Dickenson, Mountain View, Calif); and HLA DR and OKT10 (Ortho) as markers of early T cell differentiation antigens. Total numbers of these subpopulations were derived from measurements of total WBC count and differential count (for lymphocytes) by standard methods. The NK activity was assayed using radiolabeled (^{51}Cr) K562 target cells and lymphocyte effector cells at an effector–target cell (ET) ratio of 10:1 as described by Spina et al.[11]

Mitogen-induced proliferation to PHA was tested in a microassay as previously described.[12] PBMCs were cultured with a 1:100 dilution of PHA (Burroughs Wellcome) for 48 hours at 37 °C. Tritiated thymidine was added to the cultures, and cells were harvested 24 hours later and counted on a Beckman scintillation counter. Data are expressed as cpm × 10^{-3}. Skin tests to recall antigens including mumps, intermediate-strength purified protein derivative of tuberculin (PPD), and *Candida* were performed; induration of ≥5 mm after 48 hours was considered positive

Total serum immunoglobulins including IgG, IgA, and IgM were measured in the clinical chemistry laboratory at UCLA Medical Center by the laser nephelometry method (PDQ Laser Nephelometry, Hyland Laboratory, Deerfield, Ill). Circulating immunocomplexes (CIC) were measured using polyethelene glycol precipitation techniques [13]

Statistical methods. Wilcoxon rank-sum tests were used for comparisons of groups and subpopulations. Survival analyses were performed using Kaplan-Meier plots[14] and Cox proportional hazard models [15] Because levels of many immunologic parameters are being simultaneously compared, it is necessary to correct for multiple comparisons This is done using the conservative Bonferroni approach, in which the Bonferroni significance level is .05 divided by the number of comparisons. In Tables 1 through 3, *P* values of <.05 are marked with a single asterisk (*); this indicates a probable relationship, but should be viewed with some skepticism. Parameters marked with double asterisks (**) indicate *P* values that are less than the Bonferroni significance level; these should be regarded as strongly indicative of a relationship

RESULTS

The immunologic changes observed at presentation in the 97 patients with AIDS-KS showed increases of some param-

Table 1. Immunological Parameters by Tumor Stage at Initial Presentation

Immune Parameter*	Tumor Stage I n = 23	 II n = 40	 III n = 10	 IV n = 24
Total WBC	4,741 (1,726)	4,992 (2,248)	5,000 (1,700)	4,300 (1,600)
Total LYM	1,195 (559)†	1,434 (890)†	1,265 (527)†	915 (395)†
Total T cells	917 (433)	961 (629)	934 (472)	617 (377)
Total T4 (Th)	298 (260)	202 (116)	292 (242)	124 (115)
Total T8 (Ts)	555 (266)	744 (487)	594 (257)	472 (278)
T4–T8 ratio	0.58 (0.58)	0.33 (0.22)	0.54 (0.32)	0.28 (0.21)
HLA-DR (%)	32 (14)	29 (12)	22 (10)	27 (13)
OKT10 (%)	22 (18)	27 (19)	23 (19)	40 (26)
FcR (%)	16 (9)	14 (7)	13 (6)	12 (4)
NK	—	17 (10)	4 (—)	11 (5)
PHA	31 (22)	35 (24)	36 (16)	25 (24)
IgG	2,083 (1,047)	1,951 (524)	1,766 (1,267)	2,284 (1,048)
IgA	314 (188)	353 (151)	263 (85)	550 (413)
IgM	168 (82)	195 (72)	146 (40)	171 (74)
Immune complexes	95 (78)	73 (44)	258 (250)	304 (557)

Several values are underlined in columns II and IV to facilitate comparison of I and III stages with stages II and IV.

*Values for each parameter used in this and subsequent tables are expressed as follows: WBC/μL; Total blood LYM (lymphocytes) (per μL); Total T cells (per μL), Total T4 (T helper/inducer cells, Leu^3,CD4) (per μL), Total T8 (T suppressor/cytotoxic cells, Leu2$^+$, CD8) (per μL); HLA DR antigen expression [% of peripheral blood mononuclear cells (PBMCs)] T10 antigen expression (% of PBMCs); Gamma FcR+ cells (% of PBMCs) Natural killer cell (NK) ET ratio 10 1 (% of radiochromium released in control lysis); Phytohemagglutinin-induced (PHA) proliferation (Tdr cpm × 10^{-3}), IgG (mg/dL); IgA (mg/dL), IgM (mg/dL); Circulating immune complexes (μg/mL) Delayed hypersensitivity skin tests (negative for three antigens)

†Mean ± SD.

Table 2. Relationship of Immunological Parameters to Survival of AIDS-Kaposi's Sarcoma Patients with Tumor Stages I + III and II + IV and in Clinical Categories A and B

Immune Parameter*	Tumor Stage		Clinical Subtype	
	I + III	II + IV	A	B
Total LYM	78†	13	.04‡	67
Total T cells	71	16	.12	.28
Total T4	05	0001§	0001§	.32
Total T8	51	56	.86	.36
T4–T8 ratio	007‡	0001§	0003§	.12
HLA DR (%)	49	.40	45	99
OKT10 (%)	.05	.002§	.01‡	18
PHA	04‡	0001§	.003§	02‡
IgG level	.81	.05	.89	01‡
IgA level	.67	03‡	.01‡	43
IgM level	82	29	18	36
Immune complexes	.35	.0003§	.94	02‡

*Parameters for these tests are same as in Table 1.

†Numbers in the table are the P values of univariate Cox proportional hazards analysis.

‡P value <.05

§P value less than Bonferroni significance level

eters and decreases in others. In >50% of patients, decreased values (below the 5th percentile) were noted in total T4 numbers and T4–T8 ratio; increased values (above the 95th percentile) were seen in percentages of OKT10 and HLA DR cells, serum IgG, and immune complexes. In 30% to 50% of patients, decreased values of WBC, total lymphocytes, total T cells, and PHA proliferative response were observed, as were increased levels of serum IgA. In 10% to 30% of patients, decreased NK activity was documented, as was increased total T8 and serum IgM. Decreased FcR$^+$ cells were seen in <10% of patients.

Survival analysis. The important prognostic immunologic parameters at presentation (and their statistical significance) were identified as· decreased T4–T8 ratio ($P < 10^{-4}$), decreased total T4 cells ($P < 10^{-4}$), decreased PHA proliferative response ($P < 10^{-4}$), immune complexes ($P < 10^{-2}$), increased OKT10 antigen expression ($P < 10^{-4}$), and increased serum IgA level ($P < 10^{-2}$). No statistically significant correlations with survival were found for WBC count, total lymphocytes, total T cells, total T8 cells, HLA DR antigen expression, FcR$^+$ cells, NK activity, serum IgG, or IgM

The Kaplan-Meier survival curves for T4–T8 ratio, total T4 cell numbers, PHA proliferative response, and total T8 cells are shown in Fig 1. The T8 level does not correlate with survival, although the other immune parameters do

A comparison of the immunologic parameter at initial examination between those who received no treatment and those who received some form of therapy showed no significant differences. Therefore, even if the treatments prolonged life, the above survival analysis is still valid.

Relationship of immune parameters to tumor stage. The immunologic status of the patients at presentation with KS tumor stages I, II, III, and IV were compared (Table 1). Differences in the mean values of total T4 cells, T4–T8 ratio, and T10 expression are seen between the four subpopulations. These findings were confirmed on repeat analysis at the last time a blood specimen was tested for each patient. The values in Table 1 show that the order of the tumor stages that gives the closest correspondence to the seriousness of the immunologic abnormality with increasing order of severity is (I and III), II, and IV.

The importance of the immunologic parameters to patient survival was assessed by univariate proportional hazards survival analyses on two patient subpopulations. those presenting with Stage I and III tumors and those presenting with Stage II and IV (Table 2). The immunologic parameters, particularly T4–T8 ratio, total T4 cells, and PHA proliferative response have important prognostic value within the subpopulations. These immunologic measurements clearly provide independent prognostic information

Table 3. Immunologic Parameters by Clinical Level at Presentation

Immune Parameter	Clinical Level A n = 64	Clinical Level B n = 33	Difference Between A and B Levels (*P* Value)
Total WBC	4,668 (1,637)*	4,969 (2,423)	.84
Total LYM	1,199 (593)	1,313 (909)	.99
Total T-cells	849 (415)	914 (701)	.54
Total T4†	259 (198)	136 (127)	.001
Total T8	570 (302)	715 (508)	.50
T4–T8 ratio†	0.49 (0 39)	0 23 (0.23)	0001
HLA-DR (%)	28 (13)	31 (12)	.29
OKT10 (%)‡	22 (17)	38 (24)	.005
FcR (%)	14 (8)	13 (4)	.86
NK	15 (10)	13 (10)	.67
PHA‡	36 (23)	20 (19)	.02
IgG	1,997 (737)	2,405 (1,024)	.29
IgA	374 (277)	468 (298)	.12
IgM	174 (81)	183 (50)	.36
Immune complexes‡	80 (66)	281 (465)	01

*Mean (SD). Values are expressed as in Table 1

†*P* value less than Bonferroni significance level

‡*P* value <.05

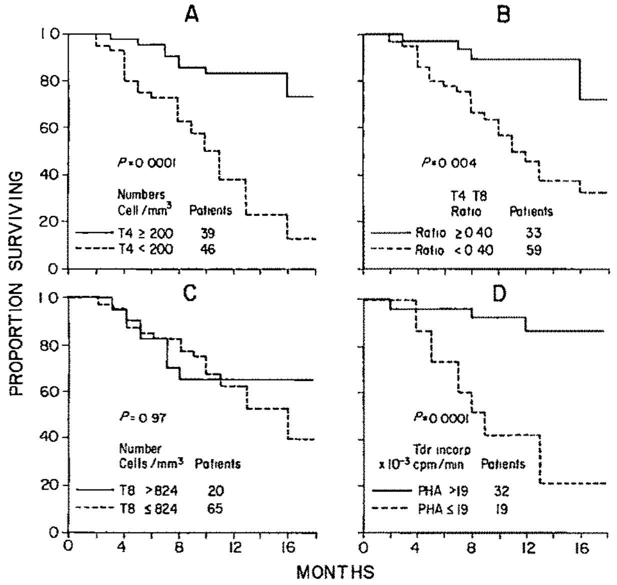

Fig 1. Relationship of survival of AIDS Kaposi's sarcoma patients to immunological parameters at the time of initial presentation: (A) T4 (CD4) cell numbers; (B) T4–T8 (CD4–CD8) ratio; (C) T8 (CD8) cell numbers; (D) proliferative response to phytohemagglutinin.

that is distinct from prognostic information based on tumor stage.

Relationship of immune parameters to clinical level. Clincal subtypes A and B differed in initial total T4 cells, T4–T8 ratio, and PHA proliferative response (all decreased), and in T10 antigen expression and serum immune complexes (both increased) (Table 3). No significant differences existed for total lymphocytes, total T cells, total T8 cells, HLA DR expression, or serum immunoglobulins. When the analysis was performed for the last time and a blood specimen was taken from the patient, the same differences between A and B clinical subtypes were seen.

The relationships of the immunologic parameters to patient survival within each clinical group were analyzed separately and found to add significant information (Table 2). Total T4 cells, T4–T8 ratio, T10 expression, and PHA proliferative response provide prognostic information for patients within clinical subtype A, indicating that an immunologic evaluation of clinical subtype A patients is useful Survival in patients with clinical subtype B, however, did not correlate with immunologic parameters. When both clinical subtype and tumor stage were combined to determine prognosis, a multivariate survival analysis revealed that total T4, T4–T8 ratio, and PHA proliferative response each gave additional survival information (data not shown) at $P <$ .002.

Skin tests were performed in 51 patients; 12 were positive responders. In tumor Stages I and III, there were 6 responders in 12 patients; in Stages II and IV there were only 6 responders in 39 patients tested. In clinical subtype classifications A and B there were 9 in 31 and 3 in 20 responders, respectively. Skin test anergy was found not to be of prognostic significance for survival ($P = .27$) The positive and negative responders did not show statistically significant differences in any of the immunological parameters considered.

Opportunistic infection. Fifteen patients had a prior or concurrent opportunistic infection (OI) at the time of diagnosis with KS. We investigated the immunologic characteristics of this group and the relationship between the immunologic parameters and the development of OI. The OI group was found to have lower T4–T8 ratios ($P < .01$), total T4 cells ($P < .01$), WBCs ($P < .01$), and total lymphocytes ($P < .05$). There were no statistically significant differences in total T8 cells, total T cells, T10 and HLA-DR antigens, PHA proliferative response, FcR cell number, NK activity, serum IgG, IgA, and IgM, or immune complexes between those with and without prior OI. The findings with respect to lower T4–T8 ratio and total T4 cell number agree with the observation that OI generally occurs with more serious immunologic impairment than does KS alone.[16]

The relationships between the immunologic parameters at initial examination with the time to future occurrence of opportunistic infections were also investigated. A univariate analysis on the time to occurrence of OI revealed that patients with lower T4–T8 ratios, total T4 cells, and PHA proliferation tended to get OIs sooner than did patients with higher ratios ($P < 10^{-4}$ for each parameter). Similarly, parameters such as T10 antigen expression ($P < 10^{-3}$), serum immune complexes ($P < .05$), and IgA ($P < .05$) related (although less well) to development of subsequent OIs. Other parameters were found not to be significant.

The above analysis shows that the occurrence of OIs is strongly related to the immunologic condition of the patient. It also indicates that immunologic abnormalities precede the development of OIs; they are not the result of OIs. The immunologic variables which are significant to OI occurrence also relate to survival; of the patients who died nearly all died as a result of OIs rather than from complications related solely to KS.

Critical immunological values. The findings noted above give strong evidence that several immunologic parameters provide valuable information in predicting the course of patients' disease. Thus, an immunologic staging of KS

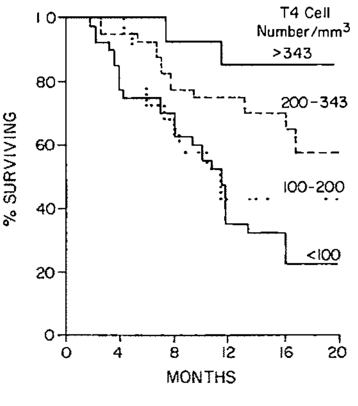

Fig 2. Relationship of various levels of T4 (CD4) cell numbers to survival in AIDS-Kaposi's sarcoma patients (Kaplan-Meier graph).

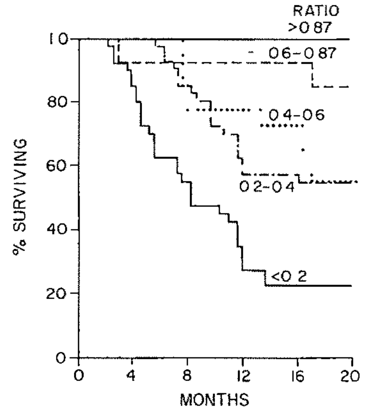

Fig 3. Relationship of various levels of T4–T8 (CD4–CD8) ratio to survival in AIDS-Kaposi's sarcoma patients (Kaplan-Meier graph).

patients should be valuable. Figures 2 and 3 show the strong relationship between T4 cell numbers and T4–T8 ratio and survival; they also show that prognosis changes rapidly as the immunologic values decrease.

By comparing survival curves for various levels of total T4 cell numbers and of T4–T8 ratio at initial examination, values indicating good, intermediate, and poor prognosis were obtained (Table 4). Eighty-five percent of patients with T4 values >300/μL and 95% of patients with T4–T8 ratio >0.5 survived 12 months, respectively. In contrast, only 35% of patients with T4 levels <100/μL and 25% of patients with T4–T8 ratio <0.2 survived 12 months. In the intermediate range of immunologic values, the prognosis changes rapidly as the values decrease. Two critical values were chosen as compromise numbers. One value would have been simpler; however, it would not have represented the large differences in survival rates accurately.

DISCUSSION

Immunologic changes relevant to prognosis are restricted to only a few parameters. Decreased T4 cell numbers and T4–T8 ratio are shown to be the most significant immunological changes in relation to prognosis and to OI development. The T4 lymphoid cell subpopulation and its reduction appears to be the central feature of AIDS. The evidence that HTLV-III is preferentially cytopathic for T4 cells[17] indicates that direct damage by the virus to T4 cells undermines the immune system; the present data point to T4 cell reduction as the key factor leading to the life-threatening complications which make AIDS such a serious disease.

Most patients with AIDS-KS are reported to have antibodies to HTLV-III[18] which is consistent with the view that HTLV-III is the etiologic agent for AIDS. Currently, available data indicate that the presence of antibodies to HTLV-III is not the key prognostic factor in AIDS-KS. Rather, the cellular immune parameters (represented in the range of T4 cell levels and T4–T8 ratios found in AIDS-KS) reflect the central pathogenic effects of HTLV-III infection and correlate with prognosis.

Immune deficiency in AIDS is manifested primarily by a deficiency of the T4 (T helper) subpopulation of lymphoid cells. Direct measurement of T4 cell numbers may provide the best prediction of prognosis. The major limitation in T4 cell quantitation is the dependence on WBC counts and differential leukocyte determinations to provide an absolute number of lymphocytes per microliter. This essential step in quantitation has far more variabilities than does the actual measurement of the percentage of T4 antigen-bearing lymphoid cells (the CD4 and T4 subpopulation)

Table 4. Critical Values of T4–T8 Ratio and T4 Cell Numbers to Prognosis

	Immunologically Defined Prognosis		
	Better	Intermediate	Poor
T4–T8 ratio	>0 5	0 5–0 2	<0.2
Surviving 12 months	>95%	(Intermediate)	25%
T4 cell numbers/μL	>300	300–100	<100
Surviving 12 months	85%	(Intermediate)	35%

Measurement of T4–T8 ratio avoids the problems associated with quantitation of lymphocytes by means of WBC and differential counts since it can be determined directly from measurements of the percentages of cells with T4 and with T8 antigens. This measurement, however, is subject to biologic variability in the level of T8 cells. The T8 cells can be changed by infections[19] and, probably, by various circumstances that have no relation to prognosis in AIDS. Our data confirm that T8 cell levels do not relate to prognosis in AIDS-KS patients. However, variation in T8 levels affects the T4–T8 ratio and reduces the precision of applying ratio measurements to AIDS prognosis.

Despite the limitations on T4 cell enumeration and T4–T8 ratio measurements, they correlate well (and better than any other parameter) with prognosis. Both measurements are recommended and can be easily made simultaneously. A repetition of the tests will also increase the value of the measurements.

These quantitative extrapolations are based on the premise documented here—that susceptibility, or lack of susceptibility, to infection is related to the quantity of T4 cells. Another immune parameter evaluated in conjunction with T4 cell numbers (or T4–T8 ratio) may improve prognostic capacity further. Such an association may also point to functional cooperation within the immune system and provide clues for better understanding of the immune system's protective functions and its potential for failure.

Measurements of T4 levels and/or T4–T8 ratio were found to have a significance that was independent of clinical status or KS tumor stage. In patients with AIDS-related complex and oral candidiasis,[20] the presence of T4–T8 ratio <0.51 has been reported to be associated with a high likelihood of developing advanced AIDS. A T4–T8 ratio >0.6 indicated a much better prognosis in these patients.

The T4 lymphoid subpopulation numbers appear normally to provide a considerable margin of protection before OIs occur. Reduction in T4 cell levels to below normal range was characteristic of AIDS-KS but was not sufficient to establish prognosis. The 5th percentile value for a normal population is more than three times greater than the critical value seen for poor prognosis in this study. Estimates of protective margin will have to be refined when the subpopulation of the T4 lymphoid cells most relevant in protection against viral, fungal, protozoal, and other infections occurring in AIDS patients has been identified.

Classical KS occurs without evidence of immune deficiency.[1-2,21] Thus, resistance or susceptibility to KS relates with less exactness than do OIs to T4 cell levels. Perhaps circumstances of host resistance or other co-factors play large roles in the initiation and development of KS.

Increases of B cell activity such as the elevated serum IgG and IgA and immune complex levels, B cell numbers in lymphoid tissues,[22] and spontaneous Ig-secreting cells,[23] are seen in many phases of AIDS. These B cell changes contrast with the reductions seen in many T cell parameters. These differences emphasize the complexity of immune cell alterations in AIDS-KS. Some of the B cell changes may reflect a lack of the immune regulation normally provided by T cells. They may also reflect either direct or indirect stimulation of B cells by HTLV-III. The B cell changes, however, do not appear to be relevant to prognosis in AIDS-KS patients. The elevation of OKT10 and HLA-DR antigens may represent an overall activation or immaturity in the lymphoid cells in AIDS patients. This question is the focus of ongoing investigations.

Clearly, the major problem in AIDS-KS is the immune deficiency. KS regressed in several transplant patients receiving immunosuppressive drugs when the immunosuppression was ended.[24,25] Correction or prevention of the T4 lymphoid cell deficiency in epidemic KS will be of prime therapeutic importance.

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Survival and Recovery of Human Platelets Stored for Five Days in a Non-Plasma Medium

By G.A. Adams, S.D. Swenson, and G. Rock

Human blood platelets were stored for five days as concentrates in 60 mL of: (a) plasma; (b) non-plasma medium with anticoagulant; and (c) non-plasma medium without anticoagulant. All preparations were equally functional when tested for platelet aggregation and release reaction in response to single agonist or synergistic pairs of agonists in vitro. Platelets stored in non-plasma medium with anticoagulant had lower kallikrein, fibrino(gen)peptide A, lactate, and β-thromboglobulin than did plasma controls after five days. In vivo recovery and survival of platelets stored in non-plasma medium with anticoagulant were 51.2% ± 4.3% and 8.7 ± 0.3 days, respectively, which were not statistically different from plasma controls of 39.2% ± 4.9% and 7.2 ± 0.8 days, respectively. It is concluded that platelets can be stored for five days in a non-plasma medium and still have good in vivo recoveries and survivals.

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BLOOD PLATELETS are routinely isolated from whole blood, concentrated, and stored in plasma for use in transfusion therapy.[1] Such platelet concentrates can be stored for five or even seven days and still be therapeutically effective in thrombocytopenic patients. Early attempts to store platelet concentrates in volumes of plasma below the normal 50 mL in polyvinylchloride bags led to a precipitous drop in pH that was associated with irreversible damage to platelets.[2-4] Thus, plasma has been considered essential for the storage of platelets for long periods. We have earlier reported on the good maintenance of in vitro platelet function when platelets were stored for three days in a modified Tyrode's buffer.[5] This report documents the storage of platelets in an infusable non-plasma medium that preserves their in vitro function for at least five days of storage, following which in vivo recovery and survival are normal.

MATERIALS AND METHODS

Materials. ^{14}C-Serotonin (5-hydroxytryptamine creatinine sulphate, Amersham Corporation, Oakville, Ontario) was diluted to 150 μmol/L in 2% ethanol in distilled water and frozen Bovine tendon collagen (SIGMA Chemical Company, St Louis) was prepared by the method of Cazenave et al[6] ADP (SIGMA) was dissolved in normal saline to 10^{-4} mol/L and frozen. The ionophore, A23187, (SIGMA) was dissolved in dimethylsulfoxide (DMSO) to 10^{-2} mol/L and then diluted to 5×10^{-5} mol/L in normal saline Epinephrine (Eastman-Kodak, Toronto) was prepared at 1.0 mmol/L in 0 15 mol/L NaCl and 1.0 mmol/L of tartaric acid, diluted to 5×10^{-4} mol/L with normal saline, and frozen Lyophilized sodium arachidonate (Bio Data Corporation, Hatboro, Pa) was reconstituted according to the company's instructions Non-plasma media were prepared by mixing concentrated sodium citrate (46 7% trisodium citrate, Haemonetics Corporation, Braintree, Mass), dextrose for injection (50%, sterile and nonpyrogenic), and Plasma-Lyte A (90 mmol/L NaCl, 23 mmol/L of sodium gluconate, 27 mmol/L of sodium acetate, 5 mmol/L of KCl, 3 mmol/L of $MgCl_2$, 294 mosm/L, pH 7.4, Travenol Canada Inc, Mississauga, Ontario). The standard non-plasma medium was composed of 59 mL of Plasma-Lyte, 0 5 mL of concentrated citrate and 0 5 mL of 50% dextrose and designated PCD One other non-plasma medium, PCD, was studied; it was free of citrate and designated PD Mixtures were made with the aid of (Millex-GS, Millipore Corp, Bedford, Mass) 0 22-μm filter units and medication injection sites

Paired studies, in vitro. Whole blood from nine normal healthy donors was collected into CPDA-1 anticoagulant. One tube was collected from each bag so that the platelets could be tested for biphasic response with epinephrine. Seven responsive units were chosen and centrifuged at 1,000 g for seven minutes in a Sorvall RC-3B at 22 °C The PRP was extracted into satellite bags and subsequently pooled and divided into six new transfer packs (Fenwal PL732, 300-mL capacity) to give 72×10^9 platelets per bag. All six units were centrifuged at 3,350 g for six minutes on the Beckman Model J-6M centrifuge, and most of the plasma was removed, leaving 7 to 9 mL The concentrated platelets were left to sit at room temperature undisturbed for one hour, after which plasma was added back to the controls and non-plasma media was added to the others. Final volumes of platelet concentrates were 60 mL. The platelet concentrates were stored at 22 °C on a vertically rotating shaker at 6 rpm (Helmer Labs Inc, St Paul) After five days, the platelets were tested for in vitro function.

Platelet function testing Platelet concentrates were diluted to 3×10^8 platelets per milliliter with either autologous plasma, non-plasma medium, or both, to standardize the aggregation conditions to three parts plasma and one part non-plasma medium After incubating with 1.0 μmol/L of ^{14}C-serotonin at 22 °C for one hour, imipramine to 2 μmol/L was added to prevent reuptake of serotonin, and platelet aggregation and the release reaction were measured Platelet stimuli were prepared at ten times final concentration and added to platelet suspensions singularly or in pairs. The following final concentrations of the aggregating agents were used epinephrine 5×10^{-5} mol/L, ADP 10^{-5} mol/L collagen 5.0 μg/mL, A23187 10^{-6} mol/L, and arachidonic acid 0.33 mg/mL. Aggregation was defined as the maximum change in transmittance after stimulation. Four minutes after stimulation, the samples were centrifuged at 15,000 g for one minute in an Eppendorf centrifuge [Brinkman Instruments (Canada), Rexdale, Ontario] to obtain supernatant samples The percentage of release of ^{14}C-serotonin was calculated as previously reported[7] Platelet and WBC counts were done on the Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, Fla) Aggregations were performed on Payton aggregometers (Payton Instruments, Toronto), and radioactivity was measured using the Beckman LS7500 liquid scintillation system (LS7500, Beckman Instruments Inc, Irvine, Calif) To illustrate the results graphically, a "synograph" was constructed by plotting the level of aggregation or release response to the various stimuli on each spoke of a 16-spoke polar graph By joining the points, an image is generated that is easily remembered and that facilitates comparison between different platelet preparations and periods of storage. The upper right quad-

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rant shows the results from single stimuli; the three other quadrants indicate the responses obtained with pairs of stimuli.

Analysis of media. The pH, pCO_2, and pO_2 of the platelet concentrates were measured with a Corning 165/2 blood gas analyzer (Corning Medical, Corning Glass Works, Medfield, Mass) at 37 °C. Glucose concentrations were determined using Fisher Diagnostics (Fair Lawn, NJ) Glucotall reagent. Lactate measurements were performed using the Boehringer-Mannheim (F.R.G.) lactate kit. Kallikrein was measured by the Diagnostica Stago (Asnieres, France) kit. β-Thromboglobulin was measured using the radioimmunoassay kit from Amersham and fibrino(gen)peptide A by the kit from Mallinckrodt (St Louis).

Autologous reinfusion studies. Autologous reinfusion studies were approved by the Medical Ethics Committee of the Ottawa General Hospital. A unit of whole blood was withdrawn from volunteers into a CPDA-1 triple pack using standard blood banking procedures. The platelets were concentrated, and as much plasma was removed as was possible. PCD was added to the platelet concentrate, which had rested undisturbed for one hour, to a final volume of 60 mL. A sample was removed for blood culture. The platelet concentrate was stored at 22 °C on a vertically rotating shaker at 6 rpm (Helmer Labs, Inc). After five days, the platelets were radiolabeled with ^{51}chromium according to recommended procedures.[8] The platelet concentrates were infused, and blood samples were collected for seven days. Platelet recovery was calculated using the average of the samples taken two and three hours after infusion; platelet survival was calculated using Murphy's multiple-hit program.

RESULTS

Two non-plasma media, PCD and PD, were used to support human platelets during storage for five days at 22 °C. The fibrino(gen)peptide A levels were greater in the PD medium with no anticoagulant than in the media, PCD, to which anticoagulant was added (Table 1). Platelet count, WBC count, pH, pCO_2, pO_2, glucose, lactate, and kallikrein were not significantly different for either non-plasma media. The plasma concentration of lactate (122 mg/dL) was twice as high as the PCD concentration of lactate (60 mg/dL). The dextrose levels were 434 mg/dL in the PCD as compared with the plasma levels of 373 mg/dL (Table 1). The kallikrein and β-thromboglobulin levels were also lower in the non-plasma medium than in the plasma after five days. Between the media and plasma, no other parameters were significantly different.

In vitro evaluation of these preparations demonstrated that platelets stored in PCD (Fig 1), PD (Fig 2), and plasma (Fig 3) had maintained their functions of aggregation and the release reaction to synergistic pairs of stimulants. Platelet responses to single stimulants were similar for PCD, PD, and plasma, but were less than the responses exhibited on the day of blood collection (data now shown). Statistical analysis of the synographs (Figs 1 through 3) were performed by calculating the paired difference for all the stimuli for both platelet responses. Platelets stored in PCD had better aggregation responses with a mean (SE) of 6% (1.3), n = 120, but had less average release of serotonin by 7.7% (1.4) compared with the plasma controls after five days of storage. Platelet concentrates stored in PD had an average 4.0% (1.0), n = 90, more aggregation and 1.8% (1.5) less release of serotonin than did plasma controls after five days of storage.

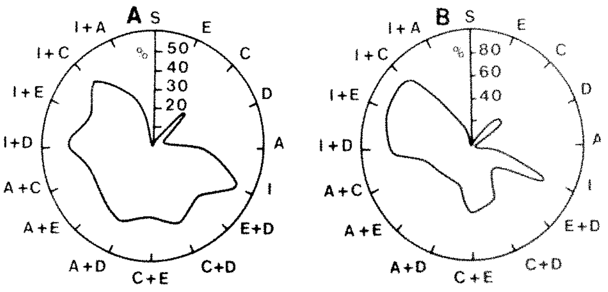

Fig 1. Synographs for platelets stored for five days in PCD (non-plasma medium). Platelet aggregation (left) and release of serotonin (right) induced by epinephrine (E), collagen (C), ADP (D), arachidonic acid (A), ionophore A23187 (I), and their binary combinations. Response is plotted as radial distance from the center of the synograph.

Autologous platelets stored for five days in the non-plasma medium and transfused into volunteers yielded excellent in vivo platelet recoveries of between 40% and 60% and mean platelet survivals of between 8.3 and 9.4 days (Table 2). The survival curves were all linear (Fig 4). The control values, reported in Table 2, were obtained with platelets stored in PL732 bags for five days as reported previously.[9] Blood cultures were negative on all preparations.

DISCUSSION

Plasma is generally not required by patients receiving platelet therapy and would be used more efficaciously if directed to fractionation. A preliminary report of the ability

Table 1. Analysis of Storage Media After Five Days of Platelet Storage at 22 °C

Medium	n	PCT 10^9/mL	WBC 10^6/mL	pH	pCO_2 mm Hg	pO_2 mm Hg	DEX (mg/dL)	LAC (mg/dL)	FPA (ng/mL)	KALL (%)	βTG (μg/mL)
Non-plasma medium											
PCD	14	1.00	2.40	7.00	21	116	434	60	2.1	32	4.4
SEM		0.01	0.30	0.03	1	4	10	2	0.7	2	2.2
PD	7	0.95	1.90	6.80	20	129	373	54	3.4	40	—
SEM		0.01	0.28	0.04	1	4	14	1	0.5	3	—
Plasma controls											
	4	0.98	2.10	7.10	30.0	130	373	122	7.8	139	6.4
SEM		0.01	0.50	0.01	0.8	4	2	2	0.8	29	1.0

n, Number of determinations; PCT, platelet count; WBC, white blood cell count; LAC, lactate; DEX, dextrose; FPA, fibrino(gen)peptide A; KALL, kallikrein concentration as percentage of normal citrated pooled plasma obtained by venipuncture and vacutainers; βTG, β-thromboglobulin.

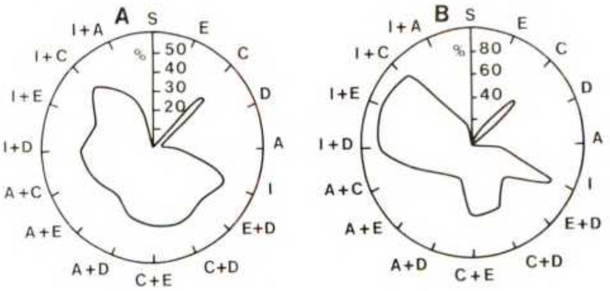

Fig 2. Synographs for platelets stored for five days in PD, (non-plasma medium without anticoagulant). Platelet aggregation (left) and release of serotonin (right) induced by stimulants (legend as in Fig 1).

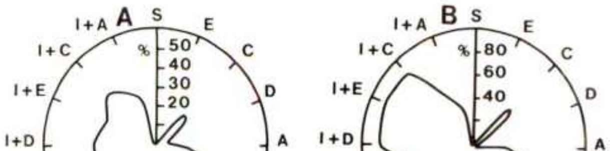

Fig 3. Synographs for platelets stored for five days in plasma (control). Platelet aggregation (left) and release of serotonin (right) induced by stimulants (legend as in Fig 1).

of non-plasma media to support platelet storage was presented by us in 1982[5] and recently confirmed by another group.[10] This report documents the ability of a non-plasma medium to support platelets adequately during five days of storage. There are many possible advantages to storing platelets in a non-plasma medium. The more precise control of the environment during storage should lead to a more consistent product. This, in turn, should lower the number of units required per infusion and hence reduce demand. The removal of plasma should diminish the risk of transfusion of infectious agents, toxins, allergens, or drugs. Last, the recovery of addditional plasma for fractionation should help prevent the adoption of expensive alternate plasma procurement procedures.

The decreased levels of lactate in the non-plasma media may offer a clue as to how the media were able to maintain platelet function so well. It has been suggested that increased oxygen transfer through the PL732 bags promotes aerobic metabolism[3] and recent analyses have suggested that the rate of lactate production is five to eight times less in well-oxygenated conditions relative to anaerobic conditions.[11] It is unlikely that the non-plasma medium could have facilitated oxygen diffusion because the bag is the limiting resistance. However, the non-plasma medium was at atmospheric oxygen tension when it was added to the concentrated platelets. This is in contrast to the situation with plasma, which is at venous oxygen levels when collected and which takes hours to days to return to atmospheric tension during storage.[9] Therefore, it may be this rapid return to high oxygen concentration on addition of the non-plasma medium that maintains aerobic metabolism and reduces lactate production during storage.

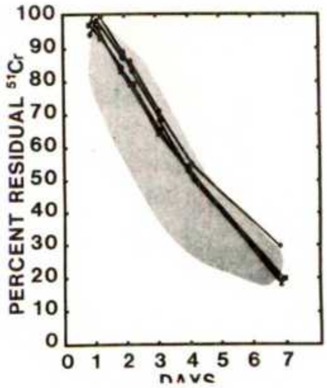

Fig 4. Platelet survival curves in four volunteers who received autologous platelets that were stored for five days in PCD (non-plasma medium). Shaded area is range of results from reinfusion of platelets stored in plasma under identical conditions.

The low levels of fibrino(gen)peptide A demonstrated that anticoagulant carried over with the residual plasma in the platelet concentrates with non-plasma medium was sufficient to guard against activation of coagulation during storage. Because there was still ~15% plasma in the platelet concentrates, this could have generated a maximum of 13,000 ng/mL (3.0 mg/mL of fibrinogen in plasma × 10,000/330,000 [mass ratio of fibrino(gen)peptide A to fibrinogen] × 15%), whereas only 3.4 to 4.4 ng/mL was detected. Therefore, although the non-plasma medium for the in vivo study (PCD) contained anticoagulant as a safety precaution, it may not have been necessary.

The decline during storage of platelet aggregation and release of dense granules in response to activation by single

Table 2. Survival and Recovery of Autologous Platelets Stored for Five Days in a Non-Plasma Medium, PCD, and Transfused Into Healthy Volunteers

		Platelet Survival (d)			
Volunteer	Linear	Weighted Mean	Multiple Hit	No. of Hits	Recovery (%)
Wi	8.4	7.8	8.4	25	40.6
Bl	8.5	8.0	8.5	25	54.0
He	8.3	7.9	8.3	25	60.9
Be	9.4	8.6	9.4	25	49.1
Mean (SE)	8.7(0.3)	8.1(0.2)	8.7(0.3)	25(0)	51.2(4.3)
Laboratory averages	7.2(0.8)		6.0(1.4)		39.2(4.9)

stimuli, but not pairs of stimuli, has been reported previously.[12] A similar pattern of dysfunction was observed in platelets after storage in the non-plasma media either with or without anticoagulant (Figs 1 through 3), implying that the acquired defects are not induced by plasma but are due to other factors, which could include contact with the plastic bag, leachables from the bags, or self-inflicted injury by the platelets themselves.

The in vivo recoveries of platelets that were stored in a non-plasma medium (PCD) were higher but not statistically different from those platelets that were stored in plasma, 51.2% *v* 39.2%, $P > .05$, respectively. The in vivo survivals were also higher but not statistically different (8.7 days *v* 6.0 days $P > .05$) for platelets stored in PCD when compared with storage in plasma. The linear nature of the survival curves (Fig 4) is indicative of the nonconsumptive removal of platelets by healthy individuals. The excellent in vivo recovery and survival of the platelets that were stored in the non-plasma medium encourages the clinical testing of these preparations in thrombocytopenic patients to demonstrate their therapeutic capacities. The similar levels of β-thromboglobulin in the plasma and non-plasma media suggest that the platelets have undergone identical degrees of activation during storage and should have equal hemostatic properties. This contention remains to be proven.

ACKNOWLEDGMENT

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Cellular *ras* Oncogene Expression and Cell Cycle Measured by Flow Cytometry in Hematopoietic Cell Lines

By Michael Andreeff, Dennis E. Slater, Jan Bressler, and Mark E. Furth

Human hematopoietic malignancies provide an excellent model for the study of the activity of cellular oncogenes in a context of known defects in cell proliferation and differentiation. A flow cytometric immunofluorescence assay was developed to quantitate the expression of the cellular *ras* oncogenes in relation to the cell cycle in individual leukemic cells. Specific binding of a monoclonal antibody to the 21-kd protein (p21*ras*) encoded by the Ha-*ras*, Ki-*ras*, and N-*ras* genes was measured by flow cytometry and confirmed by fluorescence microscopy. P21*ras* was detected in 416B, a murine hematopoietic precursor cell characterized by a high level of Ki-*ras* expression, and in the human leukemic cell lines P-12 and KG-1. The presence of p21*ras* in the cell lines was also shown by immunoprecipitation. Cellular DNA content was determined simultaneously to define cell cycle phases. There was an equal distribution of p21*ras* in G_1, S, and G_2M, with considerable heterogeneity of *ras* gene expression in the G_1 compartment. The assay allows oncogene expression to be studied in populations of intact single cells in which cell heterogeneity is maintained, requires very few cells per sample, and directly correlates oncogene expression to cell kinetic data.

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DNA ISOLATED from a wide variety of malignant cells can induce cell transformation when transfected into cultured NIH 3T3 mouse fibroblasts. In 10% to 20% of human tumors,[1] the activated transforming genes are related to the oncogenes of the Harvey or Kirsten murine sarcoma viruses or to the N-*ras* gene originally described in the human SK-S-NH neuroblastoma cell line.[2] The N-*ras* gene is of particular interest since it has been found in human T and B cell leukemia cell lines, myeloid and Burkitt's lymphoma cell lines, and in primary AML.[3-7] All three cellular *ras* genes encode proteins weighing 21,000 daltons (p21*ras*)[8,9] whose biochemical activity is the binding of guanine nucleotides.[10] H-*ras* has also been shown to have GTPase activity.[11-14] P21*ras* is localized on the inner surface of the cell membrane.[15] Point mutations in the cellular *ras* genes involving amino acid substitutions at positions 12 or 13 or 59 through 63 of p21*ras* can result in oncogenic activation.[16-21] Cell transformation has also been associated with

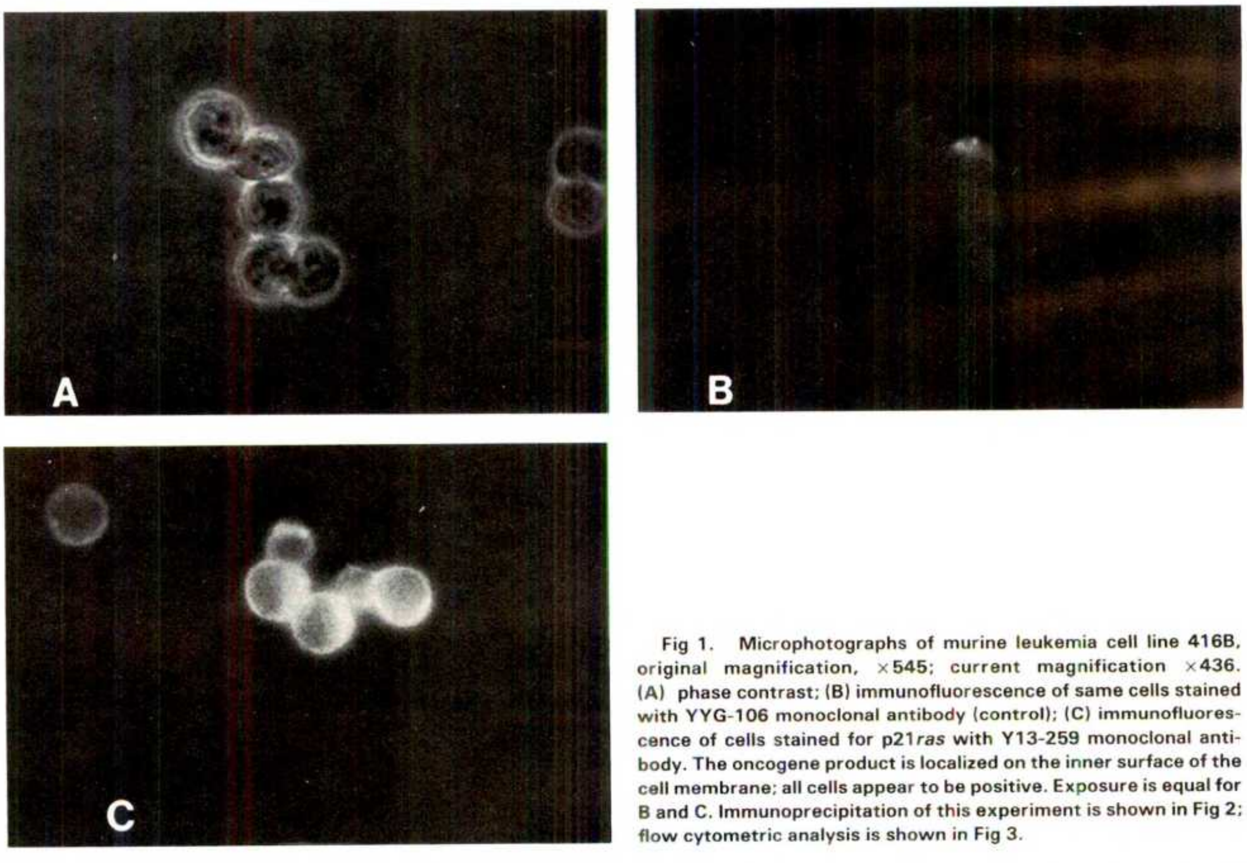

Fig 1. Microphotographs of murine leukemia cell line 416B, original magnification, ×545; current magnification ×436. (A) phase contrast; (B) immunofluorescence of same cells stained with YYG-106 monoclonal antibody (control); (C) immunofluorescence of cells stained for p21*ras* with Y13-259 monoclonal antibody. The oncogene product is localized on the inner surface of the cell membrane; all cells appear to be positive. Exposure is equal for B and C. Immunoprecipitation of this experiment is shown in Fig 2; flow cytometric analysis is shown in Fig 3.

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elevated gene expression, as in the Y1 adrenal carcinoma cell line[22] or in 416B murine hematopoietic precursor cells[23-24] in which p21 Ki-*ras* is overproduced. Similarly, the normal Ha-*ras* gene can transform NIH 3T3 cells if it is ligated to a retroviral strong transcriptional promoter.[25,26]

A flow cytometric assay of immunofluorescence in which DNA content can be measured simultaneously would be useful to determine the range of p21*ras* levels in human leukemia and lymphoma, to detect subpopulations of tumor cells with markedly high levels of p21*ras*, and to correlate *ras* gene expression with cell cycle phases. Hematopoietic malignancies provide an excellent model for the study of the activity of cellular oncogenes in a context of known defects in cell proliferation and differentiation.

MATERIALS AND METHODS

Monoclonal antibodies. A panel of rat monoclonal antibodies that precipitate p21*ras* was developed by Furth et al.[27] Y13-259 is a broad spectrum monoclonal antibody that binds p21 encoded by the Ha-*ras*, Ki-*ras*, and N-*ras* genes. YYG-106, used as a control antibody in these experiments, is a rat monoclonal antibody directed against murine hematopoietic colony-stimulating factor.

Leukemic cell lines. Cells (416B) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham F12 medium supplemented with antibiotics and 20% horse serum (Flow Laboratories, Rockville, Md). P-12 and KG-1 were grown in RPMI 1640 medium supplemented with 2 mmol/L of glutamine, 1% antibiotics and 10% heated fetal calf serum (FCS). All cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

Simultaneous staining of DNA and intracytoplasmic antigens. Leukemic cell suspensions were fixed in 100% methanol for ten minutes at −20 °C and were stained by a three-step indirect method. Fifty microliters of Y13-259 at a concentration of 50 μg/mL were incubated with 1 to 2 × 10^6 cells (minimum cell number was 0.5 × 10^6). This was followed by rabbit anti-rat IgG (Cappel, Cochranville, Pa) at a dilution of 1:200, and fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG (B&M, Indianapolis) at 1:40. All of the reagents were added for 30 minutes at 4 °C. The cells were then stained with propidium iodide after treatment with RNase as previously described.[28] The fixation and staining conditions were developed after extensive experiments (data not shown) conducted to enhance the sensitivity of the assay for the detection of low levels of p21*ras*.

A computer-interfaced research cytofluorograph (model FC201, Ortho Instruments, Westwood, Mass) with an argon-ion laser (model 75, Lexel, Palo Alto, Calif) tuned to 488 nm and operated at 30 mW was used. The signals were amplified on a linear scale. FITC green fluorescence (tertiary antibody-anti-p21*ras*), propidium iodide red fluorescence (DNA) and red pulse width (nuclear diame-

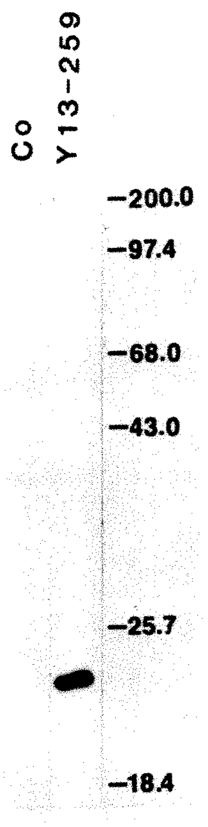

Fig 2. Radioimmunoprecipitation of murine leukemia cell line 416B with rat monoclonal antibody Y13-259 and normal rat IgG (control). The antibody precipitates p21*ras*. The same experiment is evaluated by immunofluorescence in Fig 1 and by flow cytometry in Fig 3.

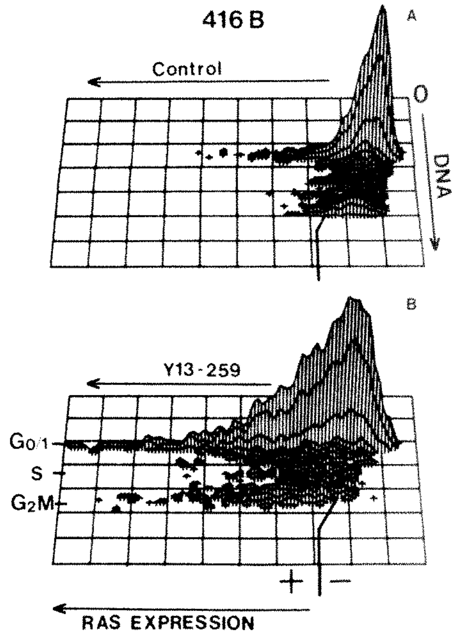

Fig 3. Computer-drawn histograms of correlated measurements of cellular DNA content and p21*ras* immunofluorescence of murine leukemia cell line 416B: *y*-axis, DNA content (cell cycle stages $G_{0/1}$-S-G_2M); *x*-axis, indirect immunofluorescence of control antibody YYG-106 (A) and Y13-259 directed against p21*ras* (B); *z*-axis, cell number. Red fluorescence (propidium iodide) pulse width was also measured simultaneously to distinguish single cells from cell aggregates (not shown). Data shown here are gated for single cells only; (−) designates negative and (+) designates positive immunofluorescence. Microphotographs of this experiment are shown in Fig 1B and C and immunoprecipitation is shown in Fig 2. DNA measurements show progression of cells from $G_{0/1}$ to S and G_2M. Almost all cells are positive for p21*ras*, with no cell cycle-related increase of *ras* expression in these exponentially growing cells. Considerable heterogeneity is apparent in all cell cycle phases, particularly in G_1.

ter) were measured in 5,000 cells per sample and stored as correlated measurements in a Nova 1220 minicomputer (Data General, Southwood, Mass). Red pulse width was used to distinguish single cells from cell aggregates. Data analysis was performed with a Tektronix 4010-1 graphic terminal (Tektronix, Beaverton, Ore) using computer programs developed by Sharpless.[29]

Immunofluorescence. Leukemic cells were stained by the three-step indirect method described above without counterstaining for DNA. Cell suspensions were mounted under coverslips with Hydromount (National Diagnostics, Somerville, NJ) and examined with appropriate filter combinations for fluorescein.

Immunoprecipitation of p21ras proteins. Cells were labeled by metabolic incorporation of ^{35}S-methionine (0.1 mCi/mL in methionine-free medium supplemented with 5% dialyzed fetal bovine serum) for five hours at 37 °C. The cells were washed and then incubated for a further period of one hour in complete medium containing unlabeled methionine and 10% fetal bovine serum. Cell lysates were prepared in a buffer containing 1% Triton X-100 (Sigma Chemical, St Louis) and 0.5% Na deoxycholate. Portions containing 10^7 cpm of incorporated ^{35}S-methionine (assessed by precipitation with trichloroacetic acid) were diluted to 0.3 mL with lysis buffer, and Na dodecyl sulfate (SDS) was added to a final concentration of 0.25% (wt/vol). One microgram of monoclonal anti-*ras* rat IgG or purified normal rat immunoglobulin was added to each tube, and reactions were incubated at 4 °C for 18 hours. Immune complexes were collected by addition of formalin-fixed *S aureus* precoated with rabbit antibodies against rat IgG, for one hour at 4 °C. The bacteria were washed extensively, and labeled proteins were eluted by boiling in sample buffer containing SDS and 2-mercaptoethanol. The samples were analyzed by electrophoresis on 15% polyacrylamide gels containing 0.1% SDS, and labeled proteins were detected by fluorography.

RESULTS

Three different cell lines were used to develop the quantitative flow cytometric assay of intracytoplasmic immunofluorescence: the murine hematopoietic precursor cell line 416B, the human myeloblastic leukemia line KG-1, and the human T cell lymphoblastic leukemia line P-12. Fig 1 shows phase contrast, control antibody YYG-106, and specific staining with anti-p21*ras* antibody Y13-259 in 416B cells. The photomicrographs were matched for magnification and for times of exposure and development. Immunoprecipitation of ^{35}S-methionine–labeled 416B cells is shown in Fig 2: Y13-259 precipitates a single band in the 21-kd region. The corresponding flow cytometric histograms are shown in Fig 3: the upper histogram shows proliferating cells in the G_1-S-G_2M phases of the cell cycle (y-axis = DNA), and defines the background fluorescence (x-axis = immunofluorescence of YYG-106). The lower histogram shows positive p21*ras* immunofluorescence in >90% of the cells. G_1 cells exhibit considerable heterogeneity, with cells expressing

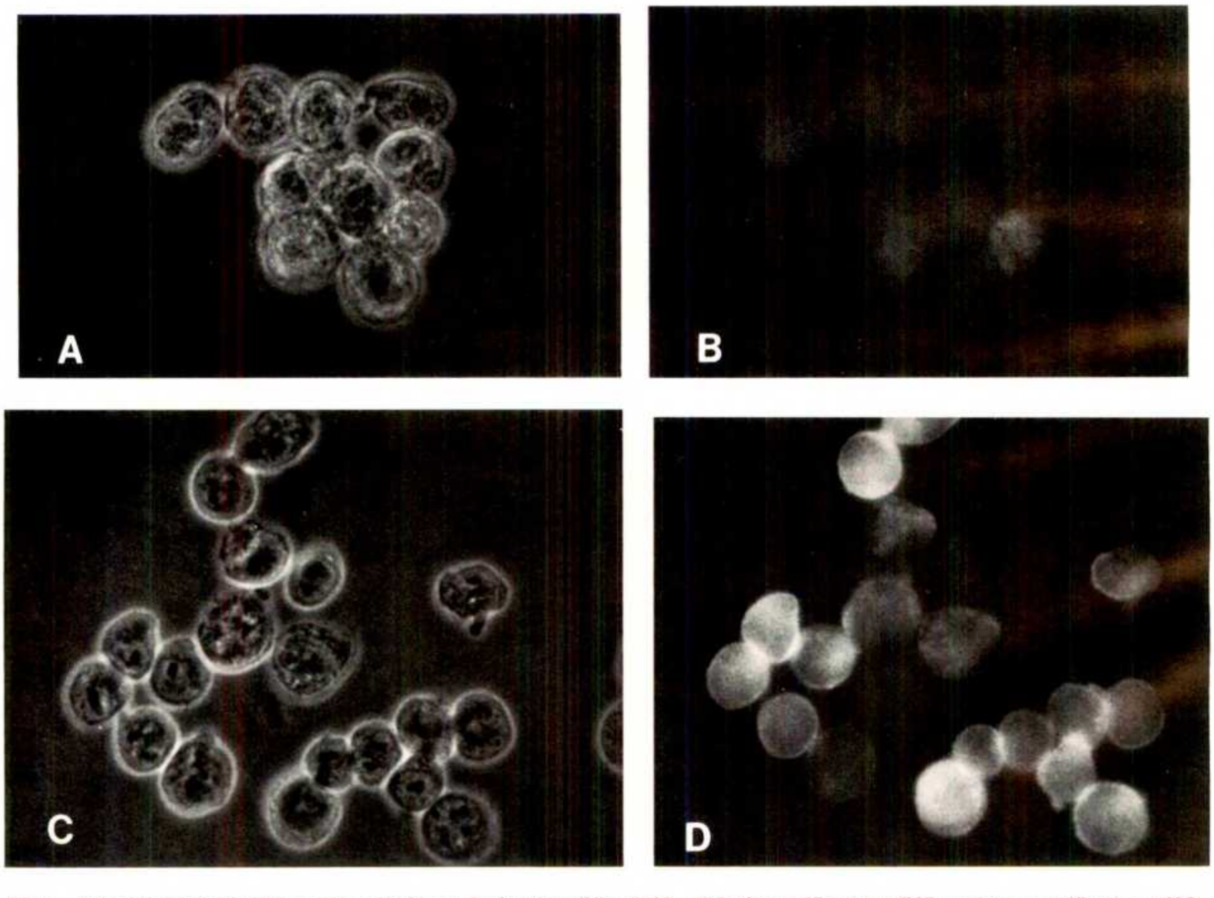

Fig 4. Microphotographs of human lymphoblastic leukemia cell line P-12, original magnification ×545; current magnification ×420. (A and C) Phase contrast of cells stained with control antibody YYG-106 (B), and anti-p21*ras* antibody Y13-259 (D), respectively. Exposure is equal for B and D. Characteristic membrane localization of p21*ras*; some cells stain as weakly as in B with control antibody. Immunoprecipitation of this experiment is shown in Fig 5, lane 1; flow cytometric analysis is shown in Fig 6.

low, intermediate, and high amounts of the cellular *ras* gene protein. There is no apparent increase in p21*ras* when the cells progress to S and G_2M if the increase in unspecific background fluorescence due to increased cell size is taken into consideration.

The human myeloid leukemic cell line KG-1 was also stained with Y13-259 and YYG-106. Approximately 60% of the cells in all cell cycle phases had fluorescence that was indistinguishable from background level. Forty percent of the cells were positive, but there was much less heterogeneity in G_1 than was found in 416B cells. Again, no increase in p21*ras* was seen during cell cycle progression (data not shown).

N-*ras*–transforming gene sequences were identified in P-12 human lymphoblastic leukemic cells in a transfection assay.[3] Fig 4 shows the typical ring pattern of p21*ras* immunofluorescence. Fig 5 depicts a one-dimensional gel in which P-12 cells were immunoprecipitated using two different monoclonal antibodies directed against p21*ras*. Y13-238 immunoprecipitates human p21 Ha-*ras* and p21 Ki-*ras* but does not immunoprecipitate p21 N-*ras* (Furth et al, unpublished observations). The band precipitated by Y13-238, corresponding primarily to p21 Ki-*ras*, was weak, but the flow cytometric assay using this antibody was positive (data not shown). A double band of p21*ras* appeared when P-12 cells were immunoprecipitated with Y13-259, since P-12 cells produce both a normal and an altered form of p21 N-*ras* with slightly different electrophoretic mobilities.[3,4] Fig 6 shows the corresponding DNA/p21*ras* histograms: ~25% of the cells in all cell cycle phases were negative, whereas the remaining cells were positive in the flow cytometric immunofluorescence assay.

The staining procedure used in these experiments yielded minimal background fluorescence, and permitted the low levels of p21*ras* found in the human leukemic cells to be detected. The assay has a greater sensitivity than the method previously described by Andreeff et al for the measurement of p21*ras* in leukemic cells.[30]

DISCUSSION

Specific binding of a monoclonal antibody directed against the protein product of the three cellular *ras* oncogenes (p21*ras*) can be measured in a quantitative flow cytometric assay of immunofluorescence. The presence of p21*ras* was confirmed by direct microscopy in three hematopoietic cell lines and by immunoprecipitation experiments using the same antibody. Oncogene expression can be directly correlated to the cell cycle since total DNA content can be determined simultaneously.

The role of p21*ras* in cell transformation and its possible relationship to the cell cycle are not completely understood.

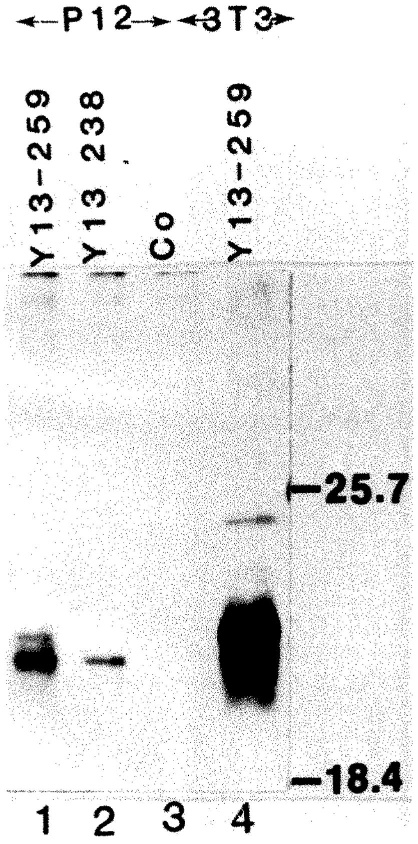

Fig 5. Radioimmunoprecipitation of human lymphoblastic leukemia cell line P-12 with antibodies against p21*ras* and control rat IgG (lanes 1 through 3). Lane 4 shows results of anti-p21*ras* antibody Y13-259 with *ras*-transfected NIH 3T3 cells.

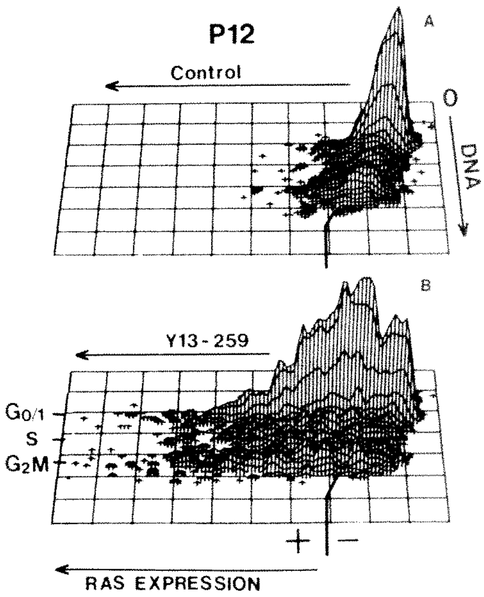

Fig 6. Computer-drawn histograms of correlated measurements of cellular DNA content and p21*ras* immunofluorescence of human lymphoblastic leukemia cell line P-12: *y*-axis, DNA content (cell cycle stages $G_{0/1}$-S-G_2M); *x*-axis, indirect immunofluorescence of control antibody YYG-106 (A) and Y13-259 directed against p21*ras* (B); *z*-axis, cell number; (–) negative and (+) positive immunofluorescence. Microphotographs of the immunofluorescence of this experiment are shown in Fig 4(B) and (D), respectively. DNA distribution shows high proliferation. Approximately 75% of cells are positive for p21*ras*, with no cell cycle related increase of *ras* expression. Heterogeneity of *ras* expression with non-Gaussian distribution is apparent in all cell cycle phases.

The ability of p21*ras* to bind guanine nucleotides[10] is probably associated with effective cell transformation. Early work by Scolnick et al showed that a temperature-sensitive mutant of the Kirsten murine sarcoma virus was temperature sensitive for transformation and that the v-Ki-*ras* protein was also temperature sensitive for guanine nucleotide binding.[31] More recently, several groups have reported that the T24 transforming *ras* protein, which has an amino acid substitution at position 12, had significantly reduced GTPase activity when compared with its normal counterpart.[11-14] Sweet et al suggested that the transforming capability of the altered protein might directly reflect prolonged stimulation of a normal cellular function whose termination signal would be the presence of GDP subsequent to GTP binding.[12] Induction of a transformed phenotype has also been associated with elevated levels of the normal *ras* protein.[22-26]

P21*ras* was found in all cell cycle phases in three hematopoietic cell lines by the flow cytometric assay. There was considerable heterogeneity in the G_1 compartment, in which some cells had a larger amount of p21*ras* than was found in any S phase cell whereas other cells were in a lower range of positivity. Cells in early and late G_1 may have different amounts of p21*ras*. It is also possible that a subpopulation requires an overproduction of p21*ras* during G_1 in order to maintain a constant level of cell proliferation. Recent studies by several investigators indicate that p21*ras* may be involved in the regulation of DNA synthesis. Campisi et al have reported that the level of c-*ras* transcripts rises in the later part of G_1 in mouse fibroblasts stimulated by the sequential action of platelet-derived growth factor, epidermal growth factor, and somatomedin C, and continues to increase as the cells enter S phase.[32] Quiescent NIH 3T3 cells growing in serum-depleted medium were transformed directly following microinjection of purified p21 Ha-*ras* and entered S phase as measured by the incorporation of ^{3}H-thymidine.[33] NIH 3T3 cells induced to divide by the addition of serum were unable to enter S if p21*ras* was neutralized by an anti-p21*ras* monoclonal antibody.[34]

The simultaneous flow cytometric assay of immunofluorescence and total DNA content described here is a powerful tool for the elucidation of the relationship between oncogene expression and the cell cycle. The assay allows oncogene expression to be measured in populations of single intact cells where cell heterogeneity is maintained, requires very few cells per sample, and directly correlates oncogene expression with cell kinetic data.

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CFU-M–Derived Human Megakaryocytes Synthesize Glycoproteins IIb and IIIa

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Human megakaryocytes have been shown by immunofluorescent techniques to express platelet glycoprotein IIb/IIIa antigen. We report evidence that megakaryocytes derived from human committed megakaryocytic progenitor cells in vitro (CFU-M) synthesize glycoproteins IIb and IIIa. Nonadherent light-density human bone marrow cells were cultured in human plasma and methylcellulose using conditions that promote large megakaryocytic colonies. On day 13 the megakaryocytic colonies were picked, pooled, and pulsed with ^{35}S-methionine in methionine-free media. Populations of approximately 100,000 cells with ≥95% viability and containing 70% to 90% megakaryocytes were obtained reliably for study. After the radioactive pulse, the cell suspension was solubilized with nonionic detergent. To reduce nonspecific binding of ^{35}S-labeled proteins to agarose, the lysate was chromatographed sequentially on glycine-quenched Affi-gel and antihuman factor X–Sepharose. The unbound material from these resins was then chromatographed on an antiglycoprotein IIb/IIIa monoclonal antibody resin (HP1-1D–Sepharose) or on a control monoclonal antibody resin. Bound fractions were eluted and analyzed by polyacrylamide gel electrophoresis and autoradiography. Autoradiograms of diethylamine eluates from HP1-1D–Sepharose revealed two labeled proteins with electrophoretic mobilities identical with those of human platelet membrane glycoproteins IIb and IIIa, isolated using similar conditions. Autoradiograms of material synthesized by control macrophages from the same donors revealed no significant labeling of proteins in the glycoprotein IIb/IIIa molecular weight range, nor were such proteins bound by HP1-1D–Sepharose. Our observations show that protein synthesis by CFU-M–derived human megakaryocytes can be readily studied using a small amount of bone marrow aspirate as starting material. This approach will allow the study of protein synthesis by megakaryocytes from normal subjects or from subjects with clinical disorders, and it will circumvent the need to obtain large amounts of bone marrow to prepare enriched populations of megakaryocytes.

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THE STUDY of protein synthesis by normal human megakaryocytes has been hampered by the difficulty in obtaining sufficiently large populations of cells markedly enriched for megakaryocytes. Other investigators have reported that von Willebrand factor, actin, coagulation factor V, and fibrinogen are synthesized by megakaryocytes enriched from guinea pig bone marrow[1-4] and that platelet factor 4 is synthesized by megakaryocytes enriched from rabbit bone marrow.[5] These studies have depended on physical separation techniques for enriching megakaryocytes directly from bone marrow such as density gradient centrifugation, sedimentation velocity, and centrifugal elutriation. Recently, the synthesis of platelet glycoproteins IIb and IIIa by human neoplastic cell lines has been reported.[6,7] Also, Rabellino et al[8] have reported preliminary evidence that megakaryocytes enriched from human marrow and placed in short-term liquid suspension culture synthesize platelet glycoproteins IIb and IIIa.

The aims of this study are to show that (1) large numbers of enriched human megakaryocytes can be obtained from colonies formed in vitro by committed megakaryocytic progenitors (CFU-M) proliferating under conditions that promote large megakaryocytic colonies and (2) these populations of megakaryocytes can be used for the study of protein synthesis. We report here the first evidence that normal human CFU-M–derived megakaryocytes can synthesize platelet glycoproteins IIb and IIIa. We believe that the linkage of in vitro culture studies of human megakaryocytopoiesis and the study of protein synthesis will help set the stage for an increased understanding of normal and disturbed megakaryocytopoiesis in vivo.

MATERIALS AND METHODS

Bone marrow culture. Bone marrow aspirates from consenting normal donors were collected into sterile glass tubes containing preservative-free sodium heparin (O'Neal, Jones and Feldman, St Louis). A mononuclear marrow cell suspension of density less than 1.077 g/mL was isolated, diluted, and plated into a plasma/methylcellulose cell culture system as previously described.[9,10]

Cells were cultured in 30% plasma from a patient with aplastic anemia and 10% conditioned medium from peripheral blood leukocytes stimulated by phytohemagglutinin (PHA-LCM). Megakaryocytic colonies were identified by inverted microscopy according to previously published criteria.[9-11] Moreover, in analyzing the cellular content of 500 picked colonies derived under these culture conditions, approximately 90% of the pooled cells were megakaryocytic as defined by Wright-Giemsa staining and by immunofluorescent staining for human coagulation factor V, platelet factor 4, von Willebrand factor, β-thromboglobulin, and platelet glycoprotein IIb/IIIa.[12,13] Cells were also cultured in 30% fetal calf serum (Hyclone, Logan, Utah) and 10% PHA-CLM to stimulate macrophage colony growth. Plating densities of 100,000 cells/mL were used to reduce contamination of colonies by surrounding cells.

On day 13 of culture, the megakaryocytic (Fig 1) or macrophage colonies were picked with a finely drawn, sterile Pasteur pipette and pooled into 200 μL of filter-sterilized methionine-free RPMI 1640 (KC-Biological, Lenexa, Kan) containing 1% bovine serum albumin and buffered with 25 mol/L HEPES at pH 7.25 (medium A). Aliquots of the pooled cells were used for cell counts, differentials after Wright-Giemsa staining, and viability determinations by trypan blue exclusion. The remainder of the pooled cells (50,000 to 150,000 cells) were diluted with 800 μL medium A and incubated for 2½ to three days at 37 °C in 5% CO_2 with 0.5 mCi ^{35}S-methionine

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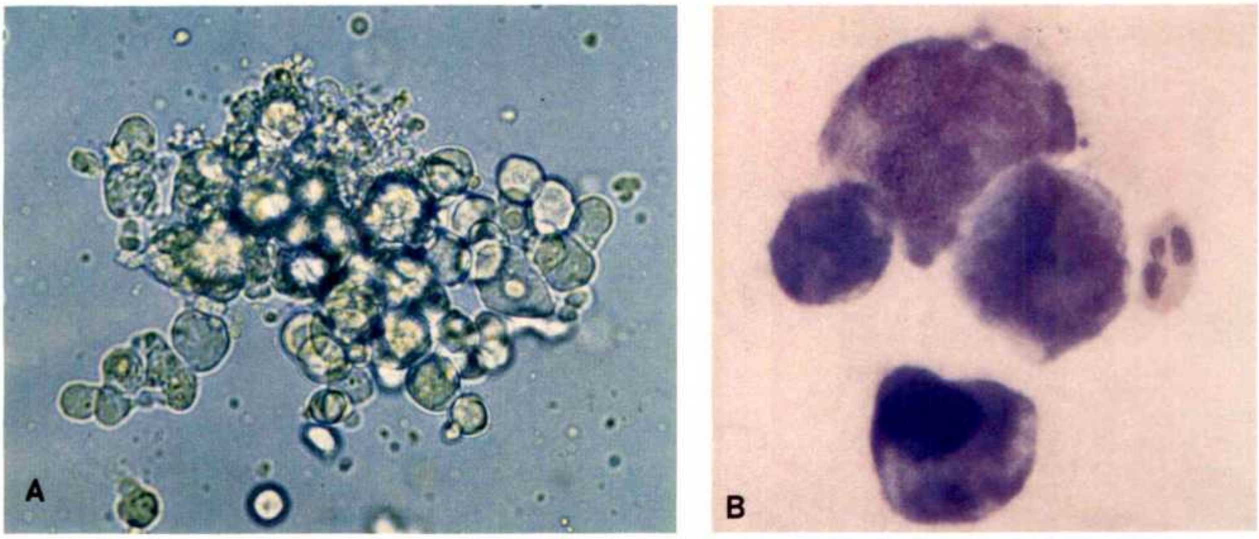

Fig 1. Typical human megakaryocytic colonies from a normal bone marrow cultured as described in Materials and Methods. (A) An in situ megakaryocytic colony visualized by inverted microscopy (original magnification ×325; current magnification ×211). (B) A picked megakaryocytic colony smeared onto a glass slide and stained by the Wright-Giemsa method (original magnification ×800; current magnification ×520). Four megakaryocytes and one neutrophil are present in the field.

(Amersham Corp, Arlington Heights, Ill; 1500 Ci/mmol). After the incubation period, the cells and medium were transferred to a glass tube coated with medium A, and Triton X-100 (J.T. Baker, Phillipsburg, NJ) was added to a final concentration of 1%. The resultant lysates were frozen at −70 °C until immunoisolation was performed.

Preparation of HP1-1D–Sepharose immunoaffinity resin. HP1-1D murine monoclonal antibody has been previously described.[13-15] The antibody is an IgG and has specificity for an epitope expressed by the human platelet membrane glycoprotein IIb/IIIa complex. HP1-1D IgG was isolated from pooled mouse ascites fluid by gel filtration chromatography[16] and was concentrated by ultrafiltration in coupling buffer (0.2 mol/L NaCl, 0.1 mol/L $NaHCO_3$, pH 8.5). CH-Sepharose 4B, activated with *N*-hydroxysuccinimide, was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Three grams of the resin were washed sequentially with 300 mL 0.001 N HCl, 300 mL water, and 30 mL coupling buffer. The washed resin was stirred for one hour at 22 °C with 13.2 mg HP1-1D IgG in coupling buffer (total volume approximately 30 mL), then 0.1 vol 1 mol/L Tris-Cl (pH 8) was added and the slurry was stirred for an additional hour before washing with approximately 5 volumes of 0.15 *M* NaCl, 0.02 mol/L Tris-Cl, pH 7.4. The resin was finally washed with approximately 5 vol of 50% glycerol, 0.01 mol/L Tris-borate, and 0.001 mol/L $CaCl_2$, pH 8.4, and was stored at −20 °C in this buffer. The efficiency of IgG coupling to activated CH-Sepharose 4B was 88% as determined spectrophotometrically (A_{280} applied − A_{280} eluted).

Immunoaffinity isolation of human platelet glycoproteins IIb and IIIa. Platelet membrane vesicles were prepared from fresh platelet-rich plasma obtained from the Mayo Clinic Blood Bank, using a modified glycerol-hypotonic lysis technique.[17] Pelleted platelet membrane vesicles (4.7 mg protein) were suspended at 22 °C in 2 mL 0.15 mol/L NaCl and 0.01 mol/L Tris-Cl, pH 7.4 (Tris-saline), which contained 1% CHAPS detergent (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Calbiochem-Behring Corp, La Jolla, Calif). Aliquots were removed from the solubilized membrane preparation before and after centrifugation (20,000 g, 15 minutes, 22 °C) for protein quantitation as described.[17] The remainder of the solubilized, centrifuged supernatant (extracted membranes that contained 72% of the membrane protein originally present) was diluted with 1 vol of Tris-saline buffer, then applied at 22 °C to a column of HP1-1D–Sepharose (plastic disposable 12-mL syringe with a porous polyethylene frit, containing 5 mL resin washed with Tris-saline and 0.1% CHAPS, pH 7.4). Following incubation (60 minutes, 22 °C), elution fractions of 2 mL were collected, using a flow rate of 1 mL/min, as the column was washed sequentially at 22 °C with approximately 20-mL vol of the following buffers: (1) Tris-saline and 0.1% CHAPS, pH 7.4; (2) Tris-saline, 0.1% CHAPS, and 5 mmol/L sodium EDTA, pH 7.4; (3) 1.5 mol/L NaCl, 0.01 mol/L Tris-Cl, and 0.1% CHAPS, pH 7.4; (4) 1.5 mol/L NaCl, 0.1 mol/L glycine-Cl, and 0.1% CHAPS, pH 3.0 (each fraction collected in 1-mL of 1 mol/L Tris-Cl, pH 8.0); (5) 3.0 mol/L NaSCN, Tris-saline, and 0.1% CHAPS, pH 7.2; (6) Tris-saline and 0.1% CHAPS, pH 7.4; and (7) 0.05 mol/L diethylamine and 0.1% CHAPS, pH 11.5 (each fraction collected in 2-mL fractions of 1 mol/L Tris-Cl, pH 8.0). Fractions were monitored spectrophotometrically (A_{280}) and by polyacrylamide gradient gel electrophoretic analysis in the presence of sodium dodecyl sulfate (SDS).[17]

For the latter analysis, pooled elution fractions were dialyzed into Tris-saline and 0.01% CHAPS and, if necessary, concentrated by ultrafiltration. Nonreduced protein samples were prepared by incubation (30 minutes, 37 °C) following the addition of *N*-ethylmaleimide to a concentration of 5 mmol/L. Prior to electrophoresis, sample preparation buffer was added to yield final concentrations of 2.5% dodecyl sulfate, 10% glycerol, 0.01% bromphenol blue, and twofold-diluted stacking gel buffer with or without 5% β-mercaptoethanol. After additional incubation (60 minutes, 37 °C), samples were electrophoresed in polyacrylamide linear gradient slab gels, and proteins were detected with Coomassie brilliant blue R-250 staining as described[17] except that (1) 1.5-mm thick slabs were used, (2) the acrylamide gradient was 4% to 12%, and (3) the lower electrode buffer consisted of 0.04 mol/L Tris-borate, pH 8.6. Gels were calibrated with reduced protein molecular weight standards obtained from Bio-Rad Laboratories (Richmond, Calif): myosin heavy chain (210,000), β-galactosidase (116,000), phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (30,000), and cytochrome c (12,500). An additional standard consisted of platelet membrane vesicle proteins treated at pH 7.4 with 5 mmol/L *N*-ethylmaleimide (30 minutes, 37 °C) prior to sedimentation in Tris-saline and solubilization (60 minutes, 37 °C) in electrophoresis sample preparation buffer (±5% β-mercaptoethanol).

Immunoaffinity isolation of megakaryocytic glycoproteins IIb and IIIa. The strategy used for the immunoisolation of megakaryocytic glycoproteins IIb and IIIa is outlined. The ^{35}S-lysates were sequentially prechromatographed on 0.5 mL of glycine-quenched Affigel-10 resin (Bio-Rad) and on 0.5 mL of mouse monoclonal antihuman factor X (HFX)-Sepharose (kindly supplied by Dr W.R. Church, Mayo Clinic) to reduce nonspecific binding of ^{35}S-labeled proteins. The resultant anti–HFX-Sepharose flow-through was divided equally into two aliquots for further immunoisolation chromatography using either HP1-1D–Sepharose (antihuman platelet glycoprotein IIb/IIIa) or control mouse monoclonal antihuman antithrombin III (HAT III) IgG coupled to Sepharose (kindly supplied by Dr M.E. Nesheim, Mayo Clinic). One-half milliliter aliquots of each resin were equilibrated with 0.02 mol/L imidazole, (J.T. Baker, Phillipsburg, NJ) 5 m*M* $CaCl_2$, pH 6.5 (Imid-$CaCl_2$). The equilibrated resins were then placed into medium A–coated glass tubes, and half of the ^{35}S-labeled flow-through from the anti–HFX-Sepharose was added to each. The tubes were sealed and placed at 22 °C on a rocking platform for 60 minutes. The mixtures were then poured into disposable minicolumns (Bio-Rad) and the flow-through material was eluted with approximately 30 mL Imid-$CaCl_2$ until the counts per minute per milliliter was less than 200. Bound materials were released stepwise using 10 mL 1.5 mol/L NaCl in Imid-$CaCl_2$, 10 mL 3.0 mol/L NaSCN in Imid-$CaCl_2$, and finally, 10 mL 0.05 mol/L diethylamine, 0.1% TritonX-100. One-milliliter fractions were collected and monitored by scintillation counting with a Beckman LS-7500 liquid scintillation counter (Beckman Instruments, Inc, Fullerton, Calif) using Beckman Redi-Solv HP as the scintillant. The diethylamine-containing fractions were immediately neutralized by the addition of 300 μL 1.0 mol/L Tris-HCl, pH 7.4. The peak fractions from each eluate were pooled, and, in experiment 1, aliquots were incubated with 5 mmol/L *N*-ethylmaleimide for 30 minutes at 37 °C. To all samples a final concentration of 0.1% SDS was added, and then the samples were dialyzed overnight *v* 0.1% SDS with one change of dialysate. All samples were then lyophilized in preparation for electrophoretic analysis.

Electrophoresis/autoradiography. Lyophilized ^{35}S-samples were dissolved in 150 μL sample preparation buffer (2% SDS; 10% glycerol; 0.06 mol/L Tris-HCl, pH 6.8; and 0.01% bromphenol blue). Reduced samples were prepared by the addition of a final concentration of 5% β-mercaptoethanol, then both reduced and unreduced samples were heated at 90 °C for ten minutes. Electrophoresis was carried out in 5% to 15% linear gradient polyacrylamide slab gels (4% acrylamide stacking gel) with the buffers described by Laemmli.[18] Approximately equivalent counts were added to each well on the slab gel. ^{125}I-protein molecular weight standards were kindly supplied by Mr G.J. Knutson (Mayo Clinic) and were used to calibrate the gels. Following electrophoresis, the gels were fixed in glacial acetic acid-ethanol-water (3:8:9, vol:vol) for 30 minutes, then placed in En^{3}Hance (New England Nuclear, Boston) for 30 minutes, followed by water rehydration for at least 45 minutes. The treated gels were mounted on chromatography paper (Bio-Rad) and dried on a slab gel drier. Each dried gel was placed inside a wafer rigid-form x-ray cassette lined with Dupont Lightning Plus intensifying screens with Kodak XAR-5 x-ray film for exposure at −70 °C. The time of exposure was adjusted according to the radioactivity of the samples applied to the gel.

RESULTS

Characterization of pooled colonies. To obtain large numbers of human megakaryocytes for the biosynthetic experiments described in the next section, CFU-M–derived megakaryocytic colonies were sterilely picked and pooled into a methionine-free serum-free medium. Table 1 summarizes the differential cell counts of pooled colonies picked from culture of marrow cells obtained from three separate normal donors. The megakaryocytic colonies were picked from 30 to 38 tissue culture plates from each donor. The average colony formation from the three donors ranged from 31 to 163 CFU-M–derived colonies per tissue culture plate (100,000 plated cells). Individual megakaryocytic colonies contained from five to 250 cells. The average number of cells per megakaryocytic colony was 105 for experiment 1, 25 for experiment 2, and 92 for experiment 3. One hundred thousand cells with 95% viability by trypan blue exclusion and consisting of 70% to 90% megakaryocytes were typically obtained. Between 5% and 16% of the cells were macrophages. Macrophages are present in the megakaryocyte population because the conditions used to support megakaryocytic colony formation also support some macrophage colony growth and some macrophages are picked accidentally. The variability in differentials between donors also reflects the donor-to-donor variation in the number, type, and density of colony growth in the cultures.

Table 1 also illustrates that macrophage colonies from the same donors can be picked and pooled with minimal megakaryocytic contamination. For control purposes in the biosynthetic experiments, the number of macrophages picked and pooled into methionine-free, serum-free medium was approximately equal to the number of contaminating macro-

Table 1. Results of Picking and Radiolabeling Megakaryocytic or Macrophage Colonies

Experiment	Suspension	Cells Picked	Mega	Macro	NRBC	PMN/Other	TCA-Precipitable cpm
1	Mega	130,000	72.3%	16.6%	5.5%	5.6%	2.70×10^7
	Macro*	30,000	0.9%	60.7%	26.6%	11.6%	1.43×10^7
2	Mega	150,000	89.1%	7.7%	3.2%	—	1.76×10^7
	Macro	15,000	1.6%	96.0%	2.4%	—	3.82×10^6
3	Mega	86,000	88.4%	5.3%	3.4%	2.8%	ND
	Macro	44,000	0.4%	95.0%	1.8%	2.8%	ND

Abbreviations: Mega, megakarocyte; Macro, macrophage; NRBC, Nucleated erythroid cell; PMN, polymorphonuclear leukocyte; ND, not determined.

*Erythropoietin (2 U/mL) was present in the medium from which the macrophage colonies were picked.

phages present in the picked and pooled megakaryocytic populations.

The picked and pooled cells from experiments 1 and 2 (Table 1) were incubated with 0.5 mCi ^{35}S-methionine for 2½ days, then the cells and medium were lysed together, and aliquots of the lysates were treated with 10% trichloroacetic acid (TCA) to determine the amount of incorporated radioactivity (Table 1). The remaining portions of the cell lysates were stored at −70 °C until used for immunoaffinity isolation of ^{35}S-labeled proteins.

Immunoaffinity isolation of platelet membrane glycoproteins IIb and IIIa. Before performing biosynthetic experiments with megakaryocytes, we characterized the conditions required for the immunofractionation of platelet glycoproteins IIb and IIIa on HP1-1D–Sepharose immunoaffinity resin. For this characterization, we used human platelet membrane preparations extracted with CHAPS detergent. Figure 2 shows the results of polyacrylamide gel electrophoresis of detergent-extracted platelet membrane vesicles immunofractionated on HP1-1D–Sepharose. As compared with the protein composition of the material applied to the HP1-1D–Sepharose column (electrophoresis lane 2), the column flow-through material (lane 3) appeared depleted of two proteins with apparent reduced molecular weights of 120,000 and 100,000. This observation suggested that HP1-1D–Sepharose bound most of the glycoprotein IIb/IIIa antigen contained in the material that was applied to the column. Subsequent washing of the column (buffers 2 through 6, Materials and Methods) did not result in the elution of significant protein (A_{280}) nor in detectable protein bands upon polyacrylamide gel electrophoresis (data not shown). The platelet membrane proteins specifically bound to HP1-1D–Sepharose were eluted with diethylamine (buffer 7, Materials and Methods). The diethylamine eluate represented approximately 12% of the protein material applied (A_{280}). Since one of the unique properties of glycoproteins IIb and IIIa is their characteristic change in electrophoretic migration after reduction of disulfide bonds,[19] we analyzed the eluted proteins before and after disulfide bond reduction. Electrophoresis of the nonreduced diethylamine eluate (lane 5) revealed two major proteins with apparent molecular weights of 135,000 and 80,000, whereas with reduction of disulfide bonds, the two proteins migrated electrophoretically with apparent molecular weights of 120,000 and 100,000, respectively (lane 4). Based upon these observations, similar immunofractionation conditions were used to study ^{35}S-labeled megakaryocytic proteins that specifically bound to HP1-1D–Sepharose.

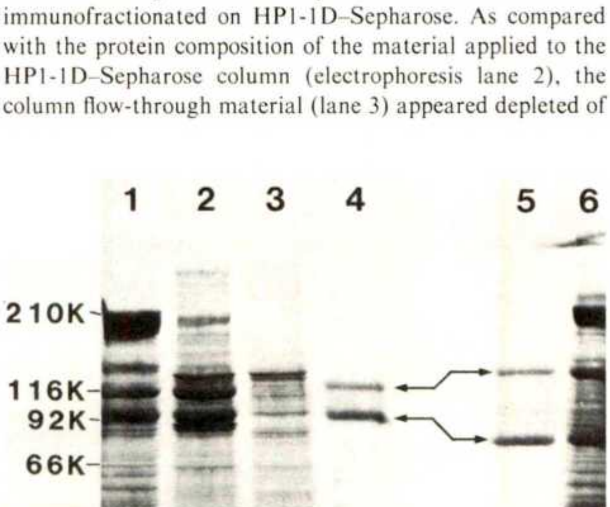

Fig 2. Polyacrylamide gel electrophoresis of immunofractionated platelet membrane proteins (Coomassie blue staining) demonstrating the specificity of HP1-1D monoclonal antibody for glycoproteins IIb and IIIa. Platelet membrane vesicles were detergent extracted and subjected to HP1-1D–Sepharose immunoaffinity chromatography. Fractions were treated with *N*-ethylmaleimide, solubilized with SDS (±β-mercaptoethanol), and subjected to 4% to 12% linear gradient polyacrylamide gel electrophoresis. The arrows between lanes 4 (reduced proteins) and 5 (nonreduced proteins) depict the electrophoretic mobilities of glycoproteins IIb and IIIa. Lanes: 1 to 4, samples reduced; 5 and 6, samples not reduced. 1, isolated platelet membranes (46 μg protein); 2, CHAPS-extracted platelet membranes (32 μg protein); 3, HP1-1D-Sepharose flow-through fraction (31 μg protein); 4, HP1-1D-Sepharose diethylamine eluate (16 μg protein); 5, HP1-1D–Sepharose diethylamine eluate (16 μg protein); and 6, isolated platelet membranes (46 μg protein).

Immunoaffinity isolation of megakaryocytic glycoproteins IIb and IIIa. Radiolabeled lysates of picked megakaryocytes were subjected to immunoaffinity chromatography as described in Materials and Methods. The lysates were prechromatographed on glycine-quenched Affigel and anti–HFX-Sepharose to decrease the amount of nonspecific binding by ^{35}S-labeled proteins to agarose. Half of the anti–HFX-Sepharose flow-through was incubated with HP1-1D–Sepharose and half with control anti–HAT III–Sepharose. Anti–HAT III–Sepharose was used as a control resin because the molecular weight of HAT III is substantially different from that of glycoproteins IIb and IIIa. Bound fractions were eluted as described in Materials and Methods, and pooled fractions were subjected to polyacrylamide gel electrophoresis and autoradiography.

Figure 3A shows the results of polyacrylamide gel electrophoresis of immunofractionated ^{35}S-labeled proteins synthesized by megakaryocytes from experiment 1. As expected, many proteins are synthesized by picked and pooled cultured megakaryocytes (lanes 1 and 13). Prechromatography on glycine-quenched Affigel and anti–HFX-Sepharose did not significantly change the megakaryocytic protein distribution (lanes 2, 3, and 8) present in the column flow-through fractions. However, two major proteins with apparent molecular weights of 116,000 and 101,000 (reduced) or 131,000 and 87,000 (nonreduced) are depleted from the HP1-1D–Sepharose flow-through (lanes 4 and 9) and are specifically released from HP1-1D–Sepharose by diethylamine (lanes 5 and 10). The apparent molecular weights (under both reducing and nonreducing conditions) of the ^{35}S-labeled megakaryocytic proteins specifically bound to and eluted from HP1-1D–Sepharose are essentially identical, within experimental error, with those of glycoproteins IIb and IIIa isolated from platelet membranes using similar conditions

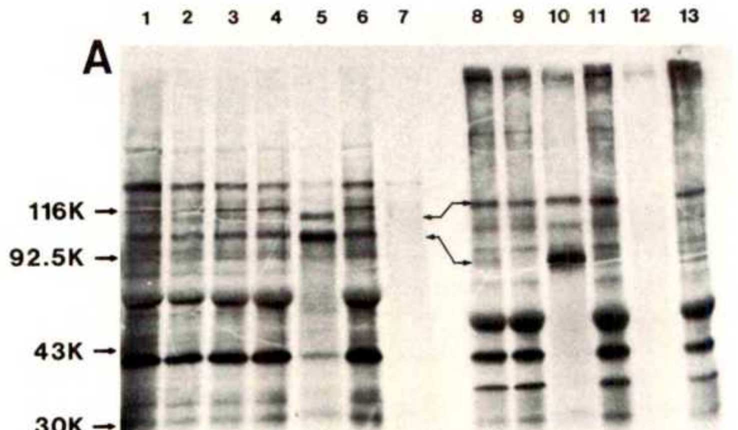

Fig 3. Polyacrylamide gel electrophoresis of immunofractionated ^{35}S-labeled proteins synthesized by human megakaryocytes and macrophages (autoradiogram) demonstrating the biosynthesis of glycoproteins IIb and IIIa by megakaryocytes. Metabolically radiolabeled megakaryocyte and macrophage proteins from experiment 1 (Table 1) were solubilized, chromatographed, and analyzed by 5% to 15% linear gradient polyacrylamide gel electrophoresis as described in Materials and Methods. (A) Picked and pooled megakaryocytes. (B) Picked and pooled macrophages. Lanes 1 to 7, samples are reduced; lanes 8 to 13, samples are not reduced. There are approximately 15,000 cpm/lane. The arrows between lanes 7 (reduced proteins) and 8 (nonreduced proteins) depict the electrophoretic mobilities of glycoproteins IIb and IIIa. Lanes: 1 and 13, solubilized cells and culture fluid; 2, Glycine-quenched Affigel flow-through; 3 and 8, anti-HFX-Sepharose flow-through; 4 and 9, HP1-1D (antiglycoprotein IIb/IIIa)-Sepharose flow-through; 5 and 10, HP1-1D-Sepharose diethylamine eluate; 6 and 11, anti-HAT III-Sepharose flow-through; and 7 and 12, anti-HAT III-Sepharose diethylamine eluate.

(Fig 2). Approximately 6.7% of the total counts per minute bound to HP1-1D–Sepharose and was eluted from the column.

Control anti–HAT III–Sepharose did not bind the two major proteins since they eluted in the flow-through fraction (lanes 6 and 11) and were not present in the diethylamine eluate (lanes 7 and 12). A protein of apparent molecular weight 42,000 under reducing conditions, probably actin, nonspecifically bound to both the HP1-1D–Sepharose and the anti–HAT III–Sepharose. This protein also nonspecifically bound to the prechromatography resins (data not shown). Under nonreducing conditions, a minor protein component with an apparent molecular weight of 105,000 was observed to bind to HP1-1D–Sepharose (lane 10). The identity of this protein is not known, but it is also a minor component of the nonreduced platelet membrane proteins that bound to and were eluted from HP1-1D–Sepharose (Fig 2, lane 5).

Since the picked and pooled megakaryocyte colonies contained a significant number of contaminating macrophages, it was important to fractionate the proteins synthesized by picked and pooled macrophages. Approximately 20,000 macrophages were treated in an identical fashion as the megakaryocytic population from the same donor. Figure 3B shows the results of polyacrylamide gel electrophoresis of immunofractionated ^{35}S-labeled proteins synthesized by the control macrophages from experiment 1. The picked and pooled macrophages were able to synthesize significant amounts of protein (Table 1 and Fig 3B, lanes 1 and 13). However, Fig 3B also shows that significant amounts of proteins with mobilities similar to glycoproteins IIb and IIIa were not synthesized by the macrophages, nor were such proteins bound by HP1-1D–Sepharose (lanes 5 and 10) or by anti–HAT III–Sepharose (lanes 7 and 12). This experiment shows that the amount of glycoproteins IIb and IIIa synthesized by the megakaryocytic population cannot be accounted for by the contaminant macrophages that were present in the megakaryocyte population.

Figure 4 shows that immunofractionation of ^{35}S-labeled proteins synthesized by megakaryocytes and macrophages from experiment 2 yielded essentially the same results as in experiment 1. Again, HP1-1D–Sepharose specifically bound

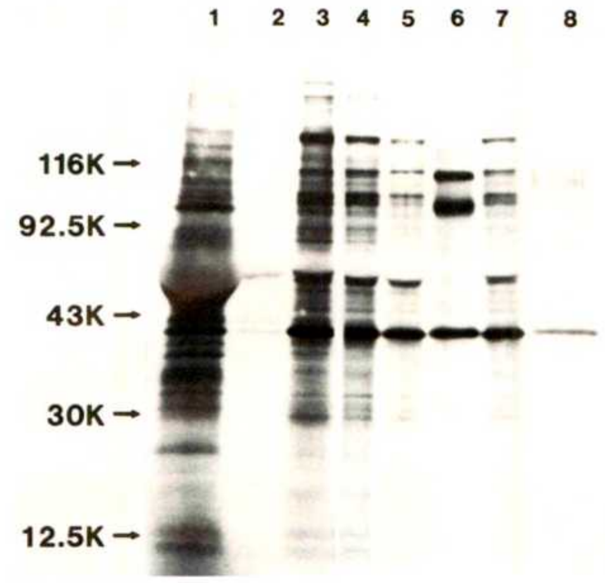

Fig 4. Polyacrylamide gel electrophoresis of immunofractionated ^{35}S-labeled proteins synthesized by human megakaryocytes and macrophages (autoradiogram), a second experiment demonstrating the biosynthesis of glycoproteins IIb and IIIa by megakaryocytes. Metabolically radiolabeled cell proteins from experiment 2 (Table 1) were solubilized, chromatographed, and analyzed by 5% to 15% linear gradient polyacrylamide gel electrophoresis as described in Materials and Methods. All samples are reduced. There are approximately 15,000 cpm/lane (except lane 2, 1,500 cpm). Lanes: 1, solubilized macrophages and culture fluid; 2, solubilized macrophages and culture fluid (1,500 cpm); 3, solubilized megakaryocytes and culture fluid; 4, megakaryocyte anti-HFX-Sepharose flow-through; 5, megakaryocyte HP1-1D-Sepharose flow-through; 6, megakaryocyte HP1-1D-Sepharose diethylamine eluate; 7, megakaryocyte anti-HAT III-Sepharose flow-through; and 8, megakaryocyte anti-HAT III-Sepharose diethylamine eluate.

and released two proteins synthesized by megakaryocytes that have apparent molecular weights (reduced) of 122,000 and 102,000 (lane 6), identical, within experimental error, to those of platelet glycoproteins IIb and IIIa. The proteins were present in the prechromatography lysate and in the anti-HFX-Sepharose flow-through (lanes 3 and 4). They were depleted in the HP1-1D-Sepharose flow-through (lane 5) and were released by diethylamine (lane 6). In experiment 2, approximately 6.1% of the applied counts per minute bound to the HP1-1D resin. Control anti-HAT III-Sepharose did not bind the two proteins since they eluted in the flow-through fraction and were not present in the diethylamine eluate (lanes 7 and 8). Again, a 42,000-dalton protein bound nonspecifically to both the HP1-1D-Sepharose and the anti-HAT III-Sepharose resins.

Approximately 15,000 macrophages from the same donor were also incubated with ^{35}S-methionine, and solubilized aliquots were electrophoresed. A volume of lysed macrophages, equivalent to the volume of lysed megakarayocytes, was electrophoresed in lane 2. No significant amount of labeled protein is seen in this sample. When tenfold more sample was electrophoresed (lane 1), many newly synthesized proteins were observed upon electrophoresis, but no intense bands were seen with mobilities similar to glycoproteins IIb and IIIa. This confirmed that the amount of glycoproteins IIb and IIIa synthesized by the megakaryocytic population in experiment 2 could not be accounted for by the number of contaminating macrophages in that population.

DISCUSSION

Our results show that protein synthesis in normal human megakaryocytes can be studied by picking and pooling CFU-M-derived megakaryocytic colonies grown in vitro. We elected to study first the synthesis of glycoproteins IIb and IIIa because of the relatively well-characterized role these proteins play in platelet hemostatic function[20,21] and because other laboratories and our own[12,13,22-24] have shown that glycoproteins IIb and IIIa are among the first proteins detected by indirect immunofluorescent techniques in the earliest stages of megakaryocytic differentiation.

We demonstrate that approximately 100,000 human megakaryocytes of 70% to 90% purity can be reliably obtained by picking and pooling CFU-M-derived megakaryocytic colonies. Other investigators have studied megakaryocytes enriched from human or nonhuman bone marrow with techniques including density gradient centrifugation and velocity sedimentation or centrifugal elutriation.[1-5,8,22,25] Both cell sorting[26,27] and panning techniques[28] have also been used to enrich marrow megakaryocytes. All of these methods either are time consuming or require relatively large amounts of bone marrow, for example, from human ribs, to obtain a significant number of megakaryocytes. Although CFU-M-derived megakaryocytes require 2 weeks of culture, only a small amount of bone marrow aspirate (2 to 3 mL) is required as starting material. In addition, when bone marrow aspiration is not clinically or technically feasible, peripheral blood mononuclear cells can serve as a source of CFU-M-derived megakaryocytes.[29] Thus, it is possible to study protein synthesis in CFU-M-derived megakaryocytes from patients with inherited or acquired disorders of platelet proteins. Our work also shows that enriched populations of other cell types, such as macrophages, can be obtained for use as control cell suspensions for synthetic studies.

Our data confirm that the murine monoclonal antibody HP1-1D reacts with the human platelet membrane glycoprotein IIb/IIIa complex.[13-15] Furthermore, HP1-1D antibody coupled to Sepharose can be used to purify platelet glycoproteins IIb and IIIa, which have appropriate nonreduced and reduced electrophoretic mobilities in polyacrylamide gels.[19] Recent studies[21] suggest that platelet membrane glycoproteins IIb and IIIa exist as calcium-dependent, noncovalently linked heterodimer complexes and that this association may be required for expression of the functional properties of the proteins. Although HP1-1D antibody is specific for glycoprotein IIb/IIIa antigen and can be used to isolate the two proteins, our studies reported here do not allow us to clearly determine whether HP1-1D antibody has specificity for a heterodimer-dependent epitope of glycoprotein IIb/IIIa or whether the epitope is present on only one of the proteins, both of which remain associated in detergent-solubilized cell preparations and coisolate using HP1-1D-Sepharose.

We find that cultured human megakaryocytes synthesize two ^{35}S-labeled proteins that are specifically bound to and released from HP1-1D–Sepharose. Similar ^{35}S-labeled proteins are not specifically bound by control anti–HAT III–Sepharose. The two synthesized proteins specifically bound by HP1-1D–Sepharose have reduced and nonreduced electrophoretic mobilities in polyacrylamide gels identical with those of platelet glycoproteins IIb and IIIa.

Our experiments do not eliminate the possibility that macrophages may synthesize small amounts of glycoproteins IIb and IIIa. However, the experiments do show that the number of macrophages contaminating the pooled megakaryocytes do not synthesize a detectable amount of glycoproteins IIb and IIIa.

The pooled megakaryocytes derived from picked megakaryocytic colonies contain cells in all stages of the megakaryocytic lineage,[12] from small, round, lymphoid-appearing cells to large megakaryocytes. Our data reported here do not allow us to make inferences about the relative synthetic capacities of early or late megakaryocytes. However, we are now subfractionating the picked and pooled megakaryocytic population to study the synthesis of proteins by cells of different size and cell surface properties.

ACKNOWLEDGMENT

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Clinical and Laboratory Characteristics of Acute Leukemia with the 4;11 Translocation

By Joseph Mirro, Geoffrey Kitchingman, Dorothy Williams, Gilles J. Lauzon, Chyi-Chyang Lin, Thomas Callihan, and Theodore F. Zipf

This report describes the clinical and laboratory features of seven cases of acute leukemia associated with the 4;11 chromosomal translocation. All seven children had acute lymphoblastic leukemia by standard morphologic and cytochemical criteria. Leukemic blasts from six of seven patients were terminal deoxynucleotidyl transferase-positive. Immunologic phenotyping suggested the leukemias were of B cell origin; blasts from five patients expressed HLA-DR and p24 (CD-9 antibody), blasts from three patients expressed B4 (CD-19), and blasts from two patients expressed the common acute lymphoblastic leukemia antigen (CD-10). One patient's leukemic blasts contained cytoplasmic immunoglobulin. Analysis of DNA from four of five patients demonstrated additional evidence of B cell differentiation with heavy-chain immunoglobulin gene rearrangement. When DNA from the four patients with heavy-chain immunoglobulin gene rearrangement was analyzed, one patient's DNA demonstrated light-chain immunoglobulin gene rearrangement. However, flow cytometric analysis of blasts from three patients showed the simultaneous expression of the lymphoid-associated antigen B4 (CD-19) and the myeloid-associated antigen My-1 (X-Hapten). Electron microscopic examination of blasts from one patient that expressed both lymphoid- and myeloid-associated antigens demonstrated ultrastructural characteristics of both lineages. These findings suggest that acute leukemia with the t(4;11) abnormality has mixed lineage characteristics as a result of leukemogenesis in a multipotential progenitor cell or aberrant gene expression later in differentiation. Furthermore, serial analysis of karyotype, immunophenotype, and heavy-chain immunoglobulin genes revealed changes in these biologic markers over time, suggesting continued chromosome rearrangement and gene modulation after the leukemogenic event in cells with the t(4;11).

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THE ASSOCIATION of the chromosomal translocation t(4;11) with acute leukemia characterized by hyperleukocytosis, a young age, and a poor prognosis has been described.[1-4] This translocation was first reported to be associated with acute lymphocytic leukemia (ALL). Further studies have suggested that the leukemogenic process involves either (1) a precursor cell with the capacity to differentiate along either myeloid or lymphoid lineages or (2) a myeloid progenitor cell.[5,6] We, therefore, analyzed the clinical and laboratory characteristics of seven patients with the t(4;11) in an effort to clarify the lineage of this abnormality. Our studies indicate that this translocation is usually associated with a lymphoid morphology and heavy-chain immunoglobulin gene rearrangement. Although the majority of blasts expressed lymphoid-associated surface antigens, careful flow cytometric analysis of cell surface antigens demonstrated the simultaneous expression of lymphoid- and myeloid-associated antigens on individual blasts from all three patients studied. Ultrastructural study in one patient confirmed dual lineage characteristics within individual leukemic cells. The expression of mixed lineage characteristics[7-11] supports the idea that either a multipotential precursor cell is involved[5,12,13] or aberrant gene expression occurs in leukemias with t(4;11).[14] Our findings are consistent with the descriptions of this translocation in both lymphoid[4] and myeloid[5,6] leukemia and in a cell line with characteristics of both lineages.[15] Furthermore, serial studies of karyotypes, immunophenotypes, and heavy-chain immunoglobulin gene rearrangements at diagnosis and relapse in our patients suggests that such biologic characteristics undergo modification in leukemic cells with this translocation.

MATERIALS AND METHODS

Patients. From Sept 1979 to Feb 1984, 329 of 413 newly diagnosed children with acute leukemia seen at St Jude Children's Research Hospital had adequate mitoses for bone marrow chromosome analysis. Six of these patients were found to have the t(4;11). From Oct 1981 to Oct 1983, 11 of 12 newly diagnosed children with acute leukemia seen at the Tom Baker Cancer Centre had adequate mitoses for bone marrow chromosome analysis, and patient 7 is from that institution. None of the patients in this series had a history of hematologic disorders. All patients were treated with modern, intensive, combination chemotherapy and CNS prophylactic therapy.[16-18] The blasts of all patients were included in leukemia cell profile studies approved by the institutions' clinical trials committees. Informed consent was obtained in each instance.

Morphologic and cytochemical studies. Bone marrow smears were stained with Wright-Giemsa, periodic-acid Schiff (PAS), myeloperoxidase (MPO), Sudan black B (SBB), chloroacetate esterase (CAE), and alphanapthyl butyrate (ANB).[19] Morphologic classification followed conventions of the French-American-British (FAB) cooperative group.[20]

Ultrastructural study. Electron microscopic studies were performed on previously frozen bone marrow cells from patient 7. Ultrastructural localization of peroxidase was performed by the method of Breton-Gorius et al.[21] The cryoprotectant was removed by washing, and the cells were fixed for one hour at 4 °C in 0.5% glutaraldehyde with 2% paraformaldehyde and 1% tannic acid. After washing, the cells were incubated in the dark at room temperature in diaminobenzidine/hydrogen peroxide for one hour.

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The cells were postfixed in 1% osmic acid, dehydrated in increasing concentrations of ethanol, and embedded in Spurr. Unstained grids and grids stained with uranyl acetate and lead citrate were examined.

Chromosome analysis. Bone marrow cells were obtained from heparinized posterior iliac crest aspirates and were processed within 30 minutes of aspiration. Direct preparations were done in all instances, and in some cases they were supplemented by 24-hour unstimulated cultures. Early studies were performed with a modification of the technique of Tjio and Whang.[22] More recently cases were studied with a direct bone marrow technique that includes treatment with trypsin and staining with Wright's stain to obtain G-banding.[23]

Immunophenotyping and terminal transferase assay. Leukemic blasts were studied for rosette formation with sheep erythrocytes at 37 °C, and the results were considered positive if rosette formation was noted for >3% of blasts (confirmed on cytocentrifuge preparations). Bone marrow samples containing >80% leukemic cells were analyzed with a standard indirect immunofluorescence assay using monoclonal antibodies (MoAb) to identify cell surface antigens. Cells were first incubated in heat-inactivated, pooled human AB serum to eliminate Fc binding. Cells incubated with isotypic myeloma immunoglobulin (Litton-Bionetics, Charleston, SC) at a concentration equal to the least diluted MoAb were used as negative controls. Lymphoid-associated antigens were identified with multiple antibodies representative of the cluster groups (CD) identified at the First or Second International Workshops on Leukocyte Differentiation Antigens and included p24 (CD-9),[24] common acute lymphoblastic leukemia antigen (CALLA) (CD-10),[25] B4 (CD-19),[26] T-11 (CD-2),[27] and T101 (CD-5).[28] The HLA-DR antigen was detected by MoAb L243.[29] When adequate cells were available, the presence of myeloid-associated surface antigens was assessed by a panel of markers including My-1,[30] Mo1 (CD-11),[31] SJ-D1 (provisional cluster, CDw14),[32] 5F1 (CDw14), and T5A7 (CDw17).[33] Immunophenotyping also included an analysis for surface (sIg) and cytoplasmic (cIg) immunoglobulins. Terminal deoxynucleotidyl transferase (TdT) was identified by use of an indirect immunofluorescence assay.[34]

Dual beam cytometric flow analysis. Two-color immunofluorescence measurements were carried out on a modified System 50H Cytofluorograf (Ortho Diagnostics, Westwood, Mass) equipped with an argon ion laser (model 164-05, Spectra Physics, Mountain View, Calif) and a tunable dye laser (model 590, Coherent, Palo Alto, Calif). The dye laser was pumped by an argon ion laser (Innova 90/5, Coherent) operating at 514 nm with an output power of 1 W. The argon ion laser operating at 488 nm (400 mW) excited fluorescein isothiocyanate conjugated (FITC) probes, whereas the dye laser was tuned to 590 nm (280 mW) for the excitation of Texas Red (TR) conjugated probes. The green (FITC) detection channel contained two bandpass (BP) filters in series: a 515-530 BP (Ortho) and a 515-525 BP (Oriel Corp, Stamford, Conn). A 625-635 BP filter (Becton Dickinson, Sunnyvale, Calif) was placed in the red detection channel. This combination of filters, in addition to the spatial separation of the images from the two beams, resulted in the complete absence of light from one fluorochrome at the detector for the other. Data was recorded and analyzed on a model 2150 computer (Ortho).

Indirect immunofluorescence was used for the determination of the presence of cell surface antigen by MoAb binding. All MoAbs were purified either by affinity or protein A–sepharose chromatography. The saturating concentration of MoAb was selected as described previously.[35] FITC goat antimouse μ- or γ-chain–specific antibodies (Cappel Laboratories, Cochranville, Pa) were used as the second-stage reagent for the green detection channel. Biotinylated MoAbs were used for analysis using the red channel, with the second-stage reagent being TR-conjugated avidin (Tago Inc, Burlingame, Calif). The application of the biotinylated antibody always followed incubation with the FITC goat antimouse step.

Two-color immunofluorescence experiments were repeated, with each primary MoAb being detected by the alternate second-stage fluorochrome to assess the consistency of the measurements. These experiments, of necessity, required that the primary MoAbs be applied in reverse order. Isotype- or subtype-identical mouse myeloma proteins were used as controls at concentrations identical to the primary MoAb as determined by an enzyme-linked immunosorbent assay. Two-dimensional arrays were then constructed using an algorithm that computed the expected number of cells in each element resulting from nonspecific binding. The array corresponding to the appropriate control antibodies was then subtracted from the array for the test antibody.

Immunoglobulin gene analysis. For each patient, 20 μg of leukemic cell DNA was digested with the appropriate enzyme *Bam*HI, *Hind*III, or *Eco*RI (3 U/μg DNA; Amersham Corp, Arlington Heights, Ill). The digested DNA was fractionated by electrophoresis on a 0.8% agarose gel, transferred to nitrocellulose paper according to the technique of Southern,[36] and hybridized with nick-translated probes with specific activities of 2 to 5 × 10^8 cpm/μg DNA. The heavy-chain immunoglobulin gene probes included a 1.2 kb *Eco*RI fragment representing portions of the $C\mu 1$ and $C\mu 3$ domains and all of the $C\mu 2$ domain[37] or a 3.4 kb *Hind*III/*Eco*RI fragment of the J_H region.[38] A 2.5 kb germ line *Eco*RI fragment was used to analyze the κ light-chain gene, and an 8.0 kb *Eco*RI fragment

Table 1. Clinical and Laboratory Findings at Diagnosis

Feature	Patient No. 1	2	3	4	5	6	7
Age (yr)	0.3	2.5	3.7	14	7	13.5	0.3
Sex	F	F	F	M	M	F	F
Leukocyte count (× 10^9/L)	201.0	27.8	27.3	345.0	12.1	346.0	276.0
Hemoglobin (g/dL)	5.8	11.7	10.0	6.0	10.2	6.6	2.3
Platelet count (× 10^9/L)	15.0	10.0	114.0	110.0	159.0	19.0	17.0
Mediastinal mass	no	no	no	no	no	no	no
CNS disease	yes	yes	no	no	no	no	yes
Liver size (cm)*	5	3	0	0	3	6	6
Spleen size (cm)*	5	0	1	0	2	9	5
FAB type	L-1	L-2	L-1	L-2	L-1	L-1	L-1

*Edge palpable below costal margin.

Table 2. Cytogenetic Results

Patient	Karyotype
1 Diagnosis	46,XX,t(4;11)(q21;q23)
Relapse	46,XX
2 Diagnosis	45,XX,−20,t(2;16)(p13;p13) 45,XX,−20,−22,+mar,t(2;16)(p13;p13)
1st Relapse	46,XX,t(4;11)(q21;q23)
2d Relapse	46,XX,t(4;11)(q21;q23),i(7q)
3 Diagnosis	48,XX,+21,+22,t(4;11)(q21;q23)
4 Diagnosis	46,XY,t(4;11)(q21;q23)
Relapse	47,XY,+X,inv(2)(p?21q31),−4,+der(4),t(4;?)(q35;?), t(4;11)(q21;q23),t(6;9)(q25;q22,t(12;19)(q23;p13)
5 Diagnosis	46,XY,t(4;11)(q21;q23),inv(16)(p13q22)
6 Diagnosis	46,XX,t(4;11)(q21;q23)
7 Diagnosis	46,XX,t(4,11)(q21;q23)
Relapse	46,XX,t(4;11)(q21;q23)

used to analyze the λ light-chain gene.[39] After high-stringency conditions were used for hybridization and washing, the filters were washed, dried, and exposed at −70 °C to XAR-5 films (Kodak, Rochester, NY) for one to five days in the presence of intensifying screens. All experiments included control DNA from normal mononuclear cells, tonsil or spleen.

RESULTS

Incidence. Of the 340 children with acute leukemia for whom chromosome analyses were performed, 237 had complete banding. The 4;11 translocation was identified in leukemic cells from six of these patients at diagnosis, indicating that the incidence of this abnormality in childhood acute leukemia is at least 2.5%.

Laboratory findings. Table 1 presents the clinical data on all seven children. The median age was 3.7 years (range, 0.3 to 14 years). Five children were female, and two were male. Three children (nos. 1, 6, and 7) had massive hepatosplenomegaly at diagnosis. The four patients (nos. 1, 4, 6, and 7) with extreme leukocytosis (median, 310 × 10^9/L; range, 210 to 346 × 10^9/L) had only the t(4;11) karyotypic abnormality detected at diagnosis (Table 2). The three patients (nos. 2, 3, and 5) with distinctly lower leukocyte counts (median, 27.3 × 10^9/L; range, 12.1 to 27.8 × 10^9/L) had associated karyotypic abnormalities and may represent a clinically distinct subgroup of patients with t(4;11). The hemoglobin concentrations (range, 2.3 to 11.7 g/dL) and platelet counts (range, 10 to 159 × 10^9/L) were variable in these patients. Three patients (nos. 1, 2, and 7) had overt CNS disease with leukemic cells in their spinal fluid. No patient had a mediastinal mass, and only one had significant adenopathy. By light microscopic examination, the leukemic blasts from all seven patients were morphologically lymphoid (FAB L1 or L2) without MPO, SBB, CAE, or ANB staining.

Six of the seven children demonstrated the t(4;11)(q21;q23) chromosomal rearrangement at the time of diagnosis, whereas patient 2 exhibited this abnormality only at relapse (Table 2). In patient 1 the t(4;11) was not detected at relapse, but patients 4 and 7 demonstrated this abnormality at both diagnosis and relapse. Three out of 4 patients had different karyotypes at relapse, and patient 2 had karyotypic changes between diagnosis, the first, and the second hematologic relapse. In four of the seven patients, the t(4;11) was associated with other chromosomal abnormalities either at diagnosis (patients 3 and 5) or at relapse (patients 2 and 4). No patient demonstrated an extra chromosome number 14 at diagnosis or at relapse.

The results of immunophenotyping and TdT analysis are presented in Table 3. A large percentage (range, 60% to 98%) of leukemic blasts from five of the six patients tested expressed TdT activity at the time of diagnosis. Blasts from all six children that could be studied at diagnosis expressed

Table 3. Immunophenotypes

	Patient No.											
			2									
	1			Relapse			4				7	
Immunophenotypes	Dx	Relapse	Dx	1st	2d	3	Dx	Relapse	5	6	Dx	Relapse
E rosettes (at 37 °C)	0	ND	1	0	0	0	0	0	ND	0	0	0
TdT	ND	87	98	25	12	90	84	6	0	60	95	ND
SIg	3	ND	1	8	10	1	0	1	ND	4	0	0
CIg	ND	ND	ND	8	6	1	48	18	ND	2	0	0
CALLA (CD-10)	ND	0	43	4	1	0	0	5	30	6	0	0
B4 (CD-19)	ND	ND	ND	ND	87	ND	85	ND	ND	89	92	95
p24 (CD-9)	ND	6	ND	72	94	85	71	60	ND	82	86	78
HLA-DR	ND	41	99	81	86	86	86	79	84	92	96	92
T-11 (CD-2)	ND	3	ND	10	0	6	2	6	ND	7	0	ND
T-101 (CD-5)	ND	21	0	13	6	3	2	4	8	2	0	ND
My-1	ND	ND	ND	26	49	ND	45	24	9	0*	23	24
Mo1 (CD-11)	ND	ND	ND	8	0	ND	ND	ND	ND	7	6	0
SJ-D1 (CDw14)	ND	0	ND	4	4	ND	3	6	2	7	ND	ND
5F1 (CDw14)	ND	ND	ND	ND	5	ND	ND	16	ND	0	ND	ND
T5A7 (CDw17)	ND	ND	ND	ND	ND	ND	55	ND	ND	61	56	62

Abbreviations: Dx, diagnosis; ND, not determined.

*Positive when tested by flow cytometry (see text).

All results given as percent positive cells determined by fluorescence microscopy.

HLA-DR, whereas four expressed p24 (CD-9). No blasts formed E rosettes or reacted with anti–T cell MoAbs. Blasts from patient 4 contained cIg at diagnosis and relapse, whereas the blasts from patient 2 expressed CALLA (CD-10) at diagnosis but not at the first or second hematological relapse. All three patients tested (nos. 4, 6, and 7) expressed the B lineage–associated surface antigen B4 (CD-19).

Cells taken at diagnosis from four patients were examined for the presence of myeloid-associated antigens. Two of these four (nos. 4 and 7) showed definite positive subpopulations (>15%). Patients 4 and 7 with My-1$^+$ blasts have relapsed and their blasts again expressed this antigen. Blasts from patient 2 were not tested at diagnosis but were My-1$^+$ at the first and second relapse. There were adequate cells remaining from the diagnostic specimens of three patients (nos. 4, 6, and 7) to allow detailed studies using flow cytometry. Using single-fluorochrome analysis, histograms demonstrated definite My-1 (X-Hapten) and T5A7 (CDw17)-positive subpopulations when compared with cells incubated with the control myeloma protein, and the ratio of My-1$^+$ cells to My-1$^+$ cells was always greater than unity (range, 1.1 to 2.3). The majority of the blasts from these three patients were also positive for the pan–B cell antigen B4 (≥85%). Dual beam immunofluorescence was then used to demonstrate that

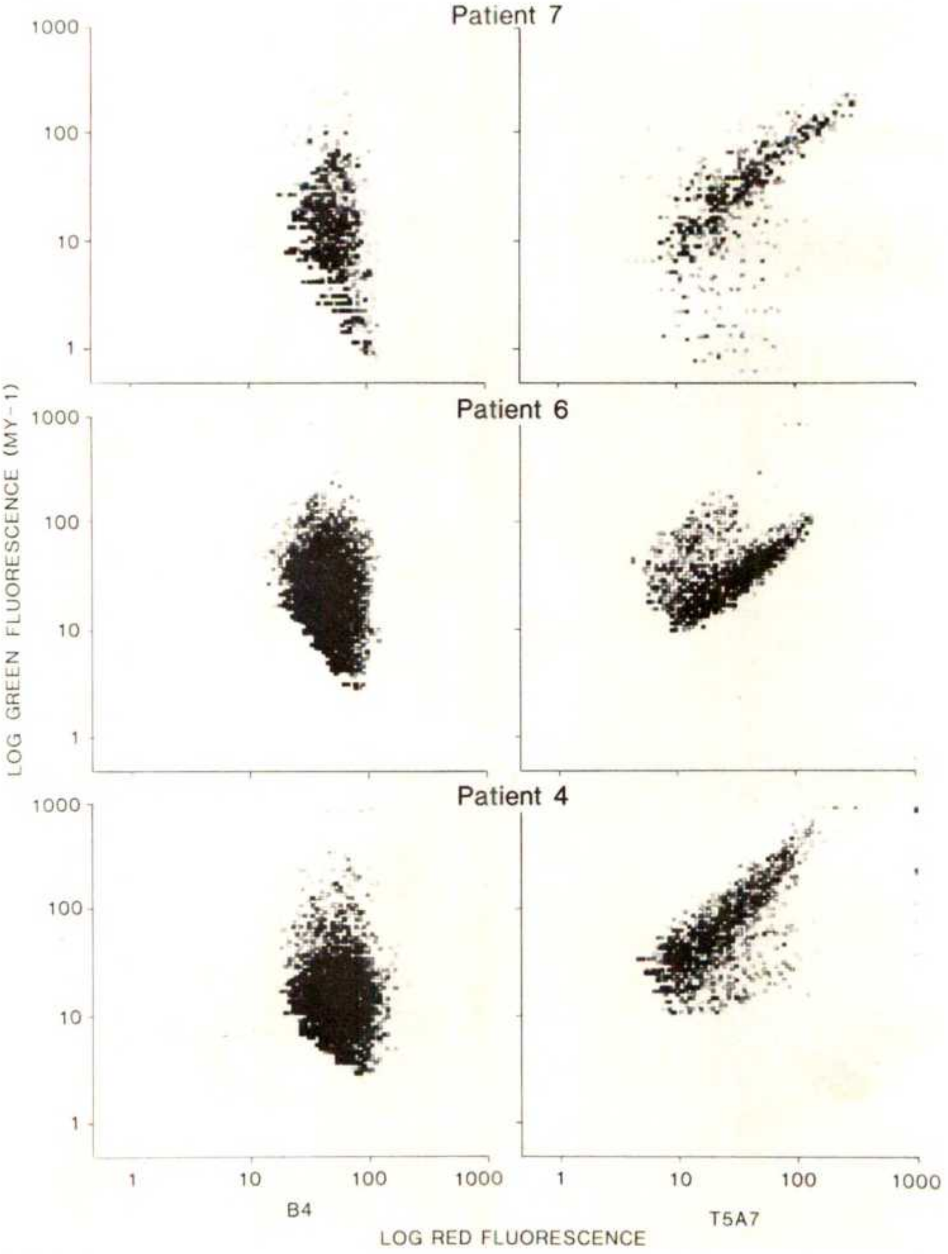

Fig 1. Results of the dual-beam immunofluorescence measurements on the My-1$^+$ blasts. The data shown here are the resultant distributions of double-labeled cells after the background resulting from nonspecific binding was subtracted. Log green fluorescence, My-1 binding, is shown on the y-axis, and Log red fluorescence, B4 or T5A7 binding, is shown on the x-axis. In all three patients (nos. 4, 6, and 7) the number of My-1$^+$ cells was equal to the number of My-1$^+$, B4+ cells and less than 10% of the My-1$^+$ cells did not bind MoAb T5A7.

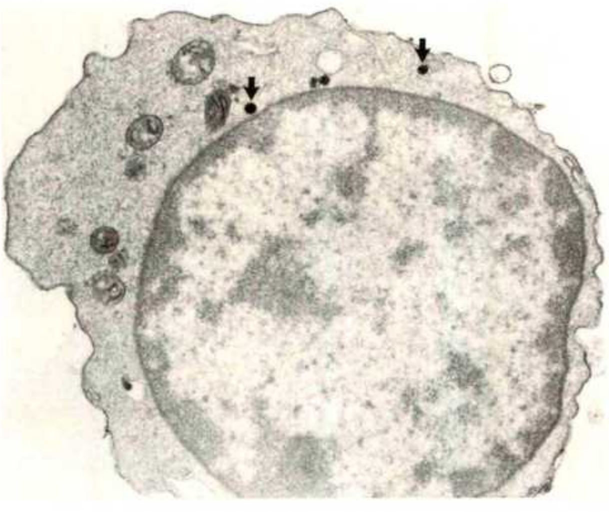

Fig 2. Electron microscopic features of leukemic blasts from patient 7. A leukemic blast with lymphoid morphology demonstrates several peroxidase-positive granules (arrows). (Original magnification ×25,475; current magnification ×9,171.)

virtually all My-1 + cells expressed B4 and greater than 90% of the My-1^{+} cells were also T5A7+ (Fig 1). The B4-positive cells also simultaneously expressed the p24 (CD-9) and HLA-DR antigens (data not shown). In the two-color experiments, the use of alternate fluorochromes, as explained in Materials and Methods, did not affect the results.

Ultrastructural studies on patient 7 were consistent with the dual-beam immunofluorescence studies. The predominant blasts appeared lymphoid with a round-to-ovoid nucleus, peripherally marginated chromatin, and one or two prominent nucleoli. The cytoplasm contained variable numbers of mitochondria, occasional short strands of rough endoplasmic reticulum, and polyribosomes. Approximately 25% of these lymphoid-appearing blasts contained electron-dense, membrane-bound granules that were peroxidase-positive (Fig 2). In addition, there was a population of blasts that was monocytoid, comprising approximately 15% to 20% of the blast cell population. These cells were larger than the lymphoid-appearing blasts and contained nuclei that were infolded with evenly distributed chromatin and a prominent nucleolus. In these cells the cytoplasm was more abundant and contained an occasional Golgi apparatus as well as numerous small electron-dense granules, some of which were peroxidase positive. Extensive examination of multiple grids failed to reveal a population of cells that exhibited dysplastic myeloid features or contained basophil/mast cell granules.

Four of five patients tested at diagnosis (nos. 1, 2, 4, and 6) showed evidence of heavy-chain immunoglobulin gene rearrangement (Table 4). Patient 6 had only a single rearranged μ gene, whereas three of these patients (nos. 1, 2, and 4) demonstrated more than two bands that hybridized to the μ constant-region probe. When the results were analyzed using a densitometer, the DNA from patient 1 had three bands (one germ line, two rearranged), and the DNA from patient 4 had at least four bands (one germ line, three rearranged) that hybridized to the Cμ probe after *Bam*HI digestion (Fig 3). Patient 2 demonstrated distinct differences in karyotype and immunophenotype between diagnosis and each relapse (Tables 2 and 3). This patient also demonstrated distinct changes in the sizes of the μ-chain gene rearrangements. When analyzed by *Bam*HI digestion, four μ constant-region–hybridizing bands were present at diagnosis, but at first hematologic relapse only three were present (Fig 3). Analysis of the DNA from patient 2 at the time of first relapse by *Hind*III digestion and J_H probing revealed a far more complex pattern than that seen by *Bam*HI digestion, with ten bands being apparent on the autoradiogram (Fig 4). Of these, 9 are rearranged and 1 is germ line. This pattern is reproducible and probably does not represent underdigestion of DNA. When DNA from the second relapse was examined by *Hind*III digestion and J_H probing, a much simpler pattern appeared, with three rearranged and one germ line μ genes being observed (Fig 4). It is clear that two of the rearranged bands represent the majority of the J_H-hybridizing material in this sample. The simultaneous analysis of the first and second relapse on the same gel clearly demonstrates conservation of some rearranged heavy-chain bands but the elimination of others. Analysis of DNA from patient 4 at the time of diagnosis using *Hind*III digestion and J_H probing also revealed a far more complex pattern than that seen using

Table 4. Immunoglobulin Gene Arrangement

Patient	Time of Study	Enzyme/Probe	μ Gene	Enzyme/Probe	κ Gene	Enzyme/Probe	λ Gene
1	Diagnosis	*Bam*HI/Cμ	$2^{+}1^{\circ}$	*Bam*HI/C$_\kappa$	1°	*Eco*RI/C	1°
	Relapse		ND		ND		ND
2	Diagnosis	*Bam*HI/Cμ	4^{+}		ND		ND
	1st Relapse	*Bam*HI/Cμ	$2^{+}1^{\circ}$	*Bam*HI/C$_\kappa$	1°	*Eco*RI/C	1°
		*Hind*III/J$_H$	$9^{+}1^{\circ}$				
	2d Relapse	*Bam*HI/Cμ	2^{+}		ND		ND
		*Hind*III/J$_H$	$4^{+}1^{\circ}$		ND		ND
3	Diagnosis		ND		ND		ND
4	Diagnosis	*Bam*HI/Cμ	$3^{+}1^{\circ}$	*Bam*HI/C$_\kappa$	1°	*Eco*RI/C	1°
		*Hind*III/J$_H$	4^{+}		ND		ND
5	Diagnosis		ND		ND		ND
6	Diagnosis	*Bam*HI/Cμ	$1^{+}1^{d}$	*Bam*HI/C$_\kappa$	$1^{\circ}1^{d}$	*Eco*RI/C	+*
7	Diagnosis	*Bam*HI/Cμ	1°	*Bam*HI/C$_\kappa$	1°	*Eco*RI/C	1°
	Relapse		ND		ND		ND

Abbreviations: 1°, germ line configuration; 1^{+}, rearranged; 1^{d}, deleted; ND, not determined.

*Several alleles are present, and one is rearranged.

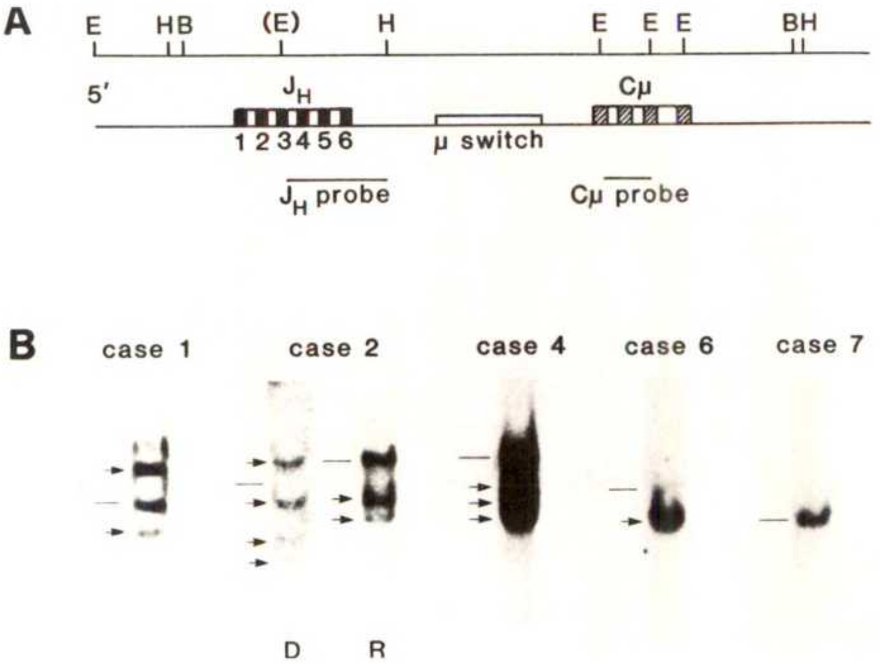

Fig 3. (A) Schematic representation of the human Ig gene region with restriction endonuclease sites: E, *Eco*RI; H, *Hind*III; and B, *Bam*HI. The Cμ probe consisted of a 1.2-kb *Eco*RI fragment subcloned into pACYC184 and contains the complete Cμ 2 domain and portions of Cμ 1 and Cμ 3. The J_H probe consisted of a 3.3-kb *Bam*HI/*Eco*RI fragment subcloned into pBR322. (B) Southern blot analysis of the Ig gene. DNA was digested with *Bam*HI, and blots were hybridized with a Cμ-specific probe. The results for cases 1, 2, (D, diagnosis; R, 1st relapse), 4, 6, and 7 are shown (lanes 1 to 6, respectively). Lines represent the positions of the 17-kb germ line gene in the gels. Arrows indicate the positions of rearranged bands (determined by densitometer).

*Bam*HI. The multiple-μ gene–hybridizing bands are clearly evident using *Hind*III digestion (Fig 4). Possible origins of the multiple bands will be discussed later.

Southern blots were washed to remove bound μ probe and rehybridized to a κ light-chain constant-region probe. None of the four patients with heavy-chain rearrangements had a rearranged κ light-chain gene (Table 4). The arrangement of the λ light-chain genes was examined using *Eco*RI digests, and one patient (no. 6) was found to have a λ light-chain gene allele rearranged. This was of interest since the κ-chain genes were apparently in the germ line configuration and κ-chain genes rearrange before λ in the Ig gene hierarchy.[40,41] Close examination of the κ-gene blot for this patient revealed that the intensity of hybridization was lower than the control DNA, indicating that perhaps one κ allele was deleted, although the second was in the germ line configuration. We conclude that, as found in several patients by Korsmeyer et al,[42] one κ-gene allele has been deleted and the rearrangement of the λ allele therefore does not violate the hierarchy of gene rearrangements.

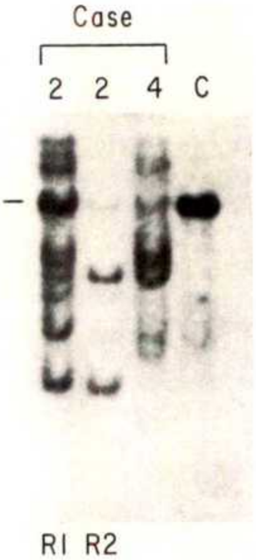

Fig 4. Southern blot analysis of the Ig gene for cases 2 and 4. DNA was digested with *Hind*III, and blots were hybridized with the J_H probe. The results for case 2 (R1, first relapse; R2, second relapse), case 4 (diagnosis), and the normal control are shown respectively in lanes 1 to 4. A line represents the position of the germ line gene.

Treatment outcome. All patients achieved complete remissions that ranged from 4+ to 30 months in duration. Patients 3, 5, and 6 remain in their first clinical remission 22+, 4+, and 4+ months, respectively. Patient 2 had a first remission that lasted 24 months and a second remission that lasted 12 months. The three patients in this report who died from progressive leukemia had survivals of 12, 14, and 41 months. Three of the four patients in whom a hematologic relapse developed achieved a second complete remission without difficulty.

DISCUSSION

We studied seven patients whose leukemic blasts had translocations between the long arms of chromosomes 4 and 11. Their ages ranged widely, and in contrast to several earlier reports,[5,6] their leukemic blast cells were clearly FAB-type L1 or L2. Only four patients had extreme hyperleukocytosis, a common finding in most series. Anemia and hepatosplenomegaly were not consistent features in our patients, and none presented with a mediastinal mass.

The majority of t(4;11)-positive patients reported in the literature achieved complete remissions; however, their remission lengths have seldom exceeded 8 months.[4-6] Whether the extended periods of complete remission in three of our patients reflect differences in other biologic characteristics or the use of more intensive therapy cannot be answered with certainty because of the small number of patients with the t(4;11).

The presence of associated karyotypic abnormalities in our patients is unusual and may be associated with important clinical characteristics such as a lower-median leukocyte count. The associated cytogenetic abnormality in patient 5 is particularly interesting since the inversion of chromosome 16 (p13q22) has been associated with nonlymphocytic leukemia.[43] The blast cells of three of four patients analyzed both at diagnosis and relapse showed karyotypic evolution, consistent with a previous report suggesting that karyotypic evolution may be common in patients with the t(4;11).[4] Not unexpectedly, we were able to show gene rearrangement and immunophenotypic evolution coincident with the observed chromosomal changes.

For the three cases in which adequate cells were available to allow extensive studies of surface antigen expression, greater than 85% of the blasts were positive for the pan–B cell antigen B4 (CD-19). This antigen is expressed early in B cell differentiation and is restricted to cells of the B lineage.[26] Subpopulations of B4$^+$ cells that expressed the p24 epitope (CD-9) also expressed the antigens identified by the MoAbs My-1 and T5A7. The molecule bearing the p24 epitope has been found on the surface of non-T, non-B ALL blasts and platelets as well as cells of monocytoid and lymphoid lineages.[24,44] MoAb My-1 detects a determinant (X-Hapten) expressed on the majority of myeloid cells and a subset of monocytes.[30,45] The reactivity of this MoAb appears to be restricted to nonlymphoid cells. The T5A7 MoAb is reactive with a lactosylceramide moiety that is the common precursor for glycosphingolipids of the lactoseries, ganglioseries, and globoseries.[46] This antibody has been shown to bind to cells of the myeloid lineage, monocytes, phytohemagglutinin-stimulated lymphocytes,[45,46] and approximately one third of non-T, non-B ALL blasts.[47] In three patients, we demonstrated simultaneous expression of a B lymphoid antigen (B4) (and several lymphoid-associated antigens) with a nonlymphoid antigen (My-1). These patients appear to have acute mixed-lineage leukemia (AMLL),[11] a finding consistent with reports of the t(4;11) in leukemic cells with lymphoid and myeloid characteristics.[4-6] A recent review of cases of hybrid acute leukemia suggested that simultaneous expression of myeloid and lymphoid characteristics may be associated with the t(4;11).[48] Of the 18 cases included in this review as hybrid leukemia, seven contained the t(4;11). Additionally, a recently described cell line containing the t(4;11) has both myeloid and lymphoid features,[15] similar to the cases we describe here.

The ultrastructural and ultracytochemical analysis of blast cells from patient 7 demonstrated both lymphoid and nonlymphoid characteristics. Thus, in this patient there appears to be a morphologic correlation with the immunologic identification of both lymphoid and nonlymphoid antigens on the same blasts. The ultrastructural features of this patient are similar to some of the previously described patients with acute leukemia characterized by the t(4;11) translocation.[5] In the series reported by Parkin et al,[5] blasts from eight of the ten patients exhibited predominantly lymphoid morphology. However, in one of the three patients tested, the lymphoid-appearing blasts contained peroxidase-positive granules.

Blasts from three of the five patients analyzed for immunoglobulin gene configuration demonstrated only heavy-chain rearrangements, whereas a fourth demonstrated both heavy- and light-chain gene rearrangements, suggesting a normal progression of B cell differentiation.[41,42,49] Although the finding of rearranged heavy-chain genes is not specific for leukemia of lymphoid lineage,[50] it is strongly B cell associated and provides indirect evidence for a lymphoid differentiation of the leukemic blasts.[41,42]

It is unusual and potentially important that three of the five patients we tested (nos. 1, 2, and 4) had greater than two DNA bands hybridizing with the immunoglobulin heavy-chain gene probe (Table 4, Figs 3 and 4). The presence of more than two rearranged μ-chain genes has been observed in transformed lymphoid cells[51] and in other leukemia patients.[52] More than two hybridizing bands could have resulted from additional alleles of the μ gene on extra copies of chromosome 14; however, we could not identify an extra chromosome 14 in any of our patients. Alternatively, multiple bands could result from more than one transformed clone, and therefore, such cases may represent biclonal leukemia.[53] A third explanation is that multiple bands are a result of evolution within the leukemic cell population. This hypothesis is supported by the finding of karyotypic evolution at relapse in the three patients having more than two heavy-chain immunoglobulin gene bands (Table 2). Further support for evolution in the leukemic population comes from our findings of coincident changes in karyotype, immunophenotype, and the heavy-chain immunoglobulin gene rearrangement at diagnosis and at relapse in one patient (no. 2). The pattern of rearrangements in patient 2 at first relapse (nine rearranged, one germ line) suggests the presence of five distinct sublines (Fig 4). This is reminiscent of the continued rearrangement of Ig heavy-chain genes that occurs in Abelson murine leukemia virus–transformed cells in which up to six sublines have been observed to arise from a clonal population over several months in culture.[54] A relationship between the evolution of biologic characteristics and relapse is purely speculative, but provocative. If such a relationship exists, it will be clarified by larger studies.[52]

In each of our cases, the break point on chromosome 11 was within or near bands q23–q24. Recent localization of the c-*ets* oncogene on chromosome 11 at this position suggests that c-*ets* could be involved in the pathogenesis of some cases of acute leukemia.[55] It will be interesting to examine t(4;11)-positive blasts for activation of the c-*ets* oncogene, which may be important for leukemogenesis.

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Biologic and Prognostic Significance of the Presence of More Than Two μ Heavy-Chain Genes in Childhood Acute Lymphoblastic Leukemia of B Precursor Cell Origin

By Geoffrey R. Kitchingman, Joseph Mirro, Sanford Stass, Ugo Rovigatti, Susan L. Melvin, Dorothy L. Williams, Susana C. Raimondi, and Sharon B. Murphy

We examined the arrangement of the μ heavy-chain immunoglobulin (Ig) genes in the leukemic blast cell DNA of 93 children with acute lymphoblastic leukemia (ALL). All cases met morphologic and cytochemical criteria for ALL, lacked detectable T cell surface antigens, and expressed HLA-DR (Ia) antigens. Eighty-three of the 93 patients (89%) were positive for the common acute lymphoblastic leukemia antigen (CALLA), and 20 of 91 (22%) tested had detectable cytoplasmic immunoglobulin. As expected, the heavy-chain Ig gene was rearranged in all cases, and the pattern of rearrangements was variable; 23 had one allele rearranged and one in the germ line configuration; 15 had one rearranged and one deleted; and 37 had two rearranged. Unexpectedly, in 18 patients the presence of more than two μ gene-hybridizing bands was detected. Combinations of enzymes and heavy-chain gene probes were used to confirm that the extra bands were not the result of underdigestion of the DNA or DNA restriction site polymorphism. In eight of the 18 patients, we identified an extra chromosome 14 as a possible cause of the extra bands' hybridizing to the μ heavy-chain constant-region probe. In the remaining ten patients, the presence of three or four bands hybridizing with the μ probe suggests the presence of two populations of leukemic cells that may have arisen either by separate leukemic transformation events or by clonal evolution of one clone into two related lines. Although preliminary (2-year follow-up), our data suggest that childhood ALL of B lineage with more than two μ heavy-chain genes, but without extra copies of chromosome 14, may be more resistant to therapy.

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EVIDENCE INDICATES that most, if not all, non-T acute lymphoblastic leukemias (ALL) are of B cell lineage. Although blasts from only 20% of children with ALL of non-T origin express cytoplasmic immunoglobulin,[1,2] blasts from all of these patients contain rearrangements in the μ heavy-chain immunoglobulin genes.[3] Approximately 40% of these ALLs also contained rearranged light-chain genes. Rearranged μ heavy-chain genes are also found in a small percentage of T-ALLs[4,5] and myeloid leukemias,[6] but in these cases, light-chain genes are not rearranged. Thus, the definitive diagnosis of the B cell lineage often depends on a combination of gene rearrangement data and monoclonal antibody markers such as common acute lymphoblastic leukemia antigen (CALLA),[1] B4,[7] and p24.[8] This allows definition not only of lineage but also of the relative level of differentiation.[3,9]

Based on analysis of karyotype,[10] G6PD isoenzymes,[11] and the idiotype of B cell lymphoma immunoglobulins,[12] leukemia and B cell lymphomas appear to be clonal diseases. Immunoglobulin gene analysis provides further support for the clonal origin of leukemias and B cell lymphomas.[13] Since oligoclonal or polyclonal tumors would exhibit either multiple bands or no specific bands, immunoglobulin gene rearrangement can be used to examine not only the cell lineage but also the clonality of a leukemia.

We examined the arrangement of immunoglobulin heavy-chain genes in blasts obtained from children with acute leukemia of B lineage and in all cases found at least one μ heavy-chain gene rearrangement, confirming the results of Korsmeyer et al.[3] Unexpectedly, we also found a number of cases with more than two bands that hybridized to the μ constant (Cμ) probe. The biologic implications of this finding for the clonal origins of ALL as well as preliminary data on the response of these leukemias to therapy are discussed.

MATERIALS AND METHODS

Diagnosis and Treatment. The diagnosis of ALL was based on standard French-American-British (FAB) morphologic and cytochemical criteria.[14] Cytochemical studies including periodic-acid Schiff (PAS), myeloperoxidase (MPO), Sudan black B (SBB), chloroacetate esterase (CAE), and alpha naphthyl butyrate (ANB) staining were performed to exclude nonlymphoid leukemias.[15] All patients were the subjects of modern, intensive multiagent chemotherapeutic protocols that included CNS prophylaxis.[16-18] All studies were approved by the institution's Clinical Trials Committee, and informed consent was obtained from the patients and their parents.

Immunologic phenotype. Immunologic studies were done on bone marrow mononuclear cells after Ficoll-Hypaque (1.077 g/mL) density centrifugation; samples routinely had greater than 80% blasts. Only leukemic blasts with an immunophenotype consistent with early B cell origin were further analyzed, whereas those suggesting T cell origin were excluded. T cell origin was defined as reactivity with T-11, T101, or OKT3, or the formation of spontaneous rosettes with sheep erythrocytes. To identify cell surface antigens using monoclonal antibodies, leukemic cells were isolated and analyzed with a standard indirect immunofluorescence assay as described previously.[19] Samples with >40% of the cells demonstrating ring fluorescence were considered positive. For these assays, cells were first incubated in heat-inactivated pooled human AB serum to eliminate Fc binding. Isotypic myeloma immunoglobulin (Litton-Bionetics, Charleston, SC) was used at a concentration equal to the least-diluted monoclone as a negative control. Immunophenotyping studies included surface (sIg) and cytoplasmic immunoglobulin

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(cIg), and lymphoid-associated antigens including p24, CALLA, and B4. The presence of terminal deoxynucleotidyl transferase (TdT) was determined by indirect immunofluorescence as previously described[20] and considered positive if greater than 40% of the cells were reactive. The HLA-DR (Ia-like) antigen was detected using the Ab279 antibody (Coulter Electronics, Hialeah, Fla).

Karyotype analysis. Chromosomes were prepared according to a direct bone marrow technique designed for ALL.[21] In each case, ten to 30 metaphases were studied for modal number and the presence of chromosome abnormalities. The karyotypes were determined by both direct microscopic analysis and photography.

Immunoglobulin gene rearrangement. Leukemic blasts were isolated from the bone marrow or peripheral blood by Ficoll-Hypaque gradients (1.077 g/mL); peripheral lymphocytes from volunteers were purified by the same procedure. DNA from lymphocytes was prepared as described by Wigler et al.[22] Twenty micrograms of DNA were digested with the appropriate enzyme, fractionated by electrophoresis on 0.8% agarose gels, and transferred to nitrocellulose paper.[23] Blots were probed with nick-translated DNA[24]; a 1.2-kb *Eco*RI fragment representing portions of the Cμ1 and Cμ3 domains and all of the Cμ2 domain of the μ heavy-chain gene,[25] a 2.5-kb germ line *Eco*RI fragment of the κ light-chain gene,[26] and a 3.4-kb *Hind*III/*Eco*RI piece of the heavy-chain joining region (J_H)[27] were used as probes. Inserts were purified from plasmids by agarose gel electrophoresis before nick-translation. After hybridization, the filters were washed at 56 °C in 0.1 × SSC (0.015 mol/L) sodium citrate, 0.15 mol/L sodium chloride, pH 7.0), 0.1% sodium dodecyl sulfate,[5] and 0.1% sodium dodecyl sulfate[5] and exposed to Kodak XAR film in the presence of intensifying screens.

RESULTS

μ heavy-chain immunoglobulin gene analysis in non-T ALL. The DNA from marrow or peripheral lymphoblasts of 93 children with non-T ALL was examined for the presence of rearranged immunoglobulin heavy-chain genes. All the patient samples were diagnostic of ALL, with typical lymphoblastic morphology and negative myeloid-associated cytochemistries (MPO, SBB, CAE, and ANB). Eighty-three of the patients expressed CALLA. Of the ten patients with blasts that did not express CALLA, six of the six tested expressed p24, all three tested expressed B4, and one patient's blasts contained cIg. At least one rearranged μ allele was found in all cases (Table 1). Twenty-three of the patients had one μ gene in the germ line configuration and one rearranged, whereas 37 showed rearrangement of both alleles. Fifteen patients demonstrated only one rearranged allele, indicating that the second allele had been deleted. Unexpectedly, 18 patients (19.4%) had more than two bands hybridizing to the Cμ probe.

Clinical and laboratory characteristics of patients with greater than two μ gene bands. The clinical and biologic characteristics of 18 ALL patients with greater than two bands hybridizing with the heavy-chain gene probe are summarized in Table 2. The median age was 3.4 years (range, 1.3 to 13.7 years), and there were 12 girls and 6 boys in this group. The median initial circulating leukocyte count was 36.2×10^9/L (range, 1.8 to 345.0×10^9/L), higher than might be expected for a group of children with early B lineage leukemia, but not significantly different statistically by the two-tailed *t* test from the other 75 patients studied ($P = .38$). Sixteen of the 18 patients were classified as FAB-L1 and two as L2. Of the 17 patients tested, 14 expressed TdT.

Table 1. Arrangement of Immunoglobulin μ Heavy-Chain Genes in ALL

Type of ALL	1°	$1^{+}1^{\circ}$	$1^{+}1^{d}$	2^{+}	>2	Total
CALLA$^-$	0	3 (27%)	2 (18%)	3 (27%)	3 (27%)	11
CALLA$^+$	0	20 (24%)	13 (16%)	34 (41%)	15 (18%)	82

Abbreviations: $^{\circ}$, germline configuration; ^{d}, deleted allele; $^{+}$, rearranged allele.

None of the patients blasts expressed T cell–associated surface antigens. Blasts from all 17 patients tested were HLA-DR (Ia) positive, and 14 expressed CALLA. Blasts from the three CALLA$^-$ patients expressed p24. Of the 17 patients from this group examined for cIg, six (35%) were positive.

Cytogenetic analysis. The karyotypes of all 18 patients are shown in Table 3. The modal number of the karyotypes at the time of diagnosis was 45 ($n = 1$), 46 ($n = 6$), 47 to 49 ($n = 2$), and >50 ($n = 9$). Eight of the nine patients with more than 50 chromosomes had an extra copy of chromosome 14 (patients 3, 8, 13 to 18). None of the remaining patients with less than 50 chromosomes had an extra chromosome 14. Numerous structural abnormalities, including six reciprocal translocations, were present in these cases, but none involved chromosome 14. Three patients contained marker chromosomes that have neither the size nor the banding pattern of a normal chromosome 14. In none of the karyotypes is there clear evidence of multiple independent clones. However, in one of the patients (no. 2), there were two related lines.

Heavy-chain gene rearrangements in patients with more than two μ gene bands. A representative Southern blot with DNA from eight of the patients is shown in Fig 1. Four of the patients (1, 2, 6, and 7) had four bands that hybridized with the μ probe, and the others demonstrated three μ-hybridizing bands (patients 3, 4, 5, and 8). The presence of a germ line μ-gene band in patients 3 and 5 cannot be due to the presence of normal cells because the percentage of contaminating normal cells in the preparation was significantly below the percentage of germ line DNA as determined by scanning densitometry (data not shown). The detection of cIg indicates that productive rearrangements had occurred in patients 5, 7, and 8.

To eliminate the possibility that the extra bands represent the presence of a polymorphic *Bam*HI site in these patients, nine of the patients' DNAs were examined further with *Hind*III digestion and J_H probing, and four were further examined by *Eco*RI digestion and J_H probing. The results, summarized in Table 4, were always consistent with the *Bam*HI digestion and Cμ probing results. These experiments eliminate other explanations for the extra Cμ-hybridizing bands, such as incomplete digestion of the DNA or somatic mutation.

Light-chain gene rearrangement. Sufficient DNA was available from 11 of the 18 patients to examine the arrangement of the κ light-chain genes. A κ constant-region probe demonstrated a germ line configuration for nine of the 11 DNAs (Table 4), with the remaining two DNAs (18.2%)

Table 2. Clinical and Laboratory Characteristics of Patients With Greater Than Two μ Gene Bands

	Patient No.																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Age (yr)	2.8	3.2	2.3	7.5	2.3	3.3	13.7	4.7	8.3	6.3	2.9	1.3	3.4	6.1	4.3	3.5	8.4	1.9
Race/sex	W/F	W/F	W/M	W/F	W/M	B/F	B/M	W/F	W/F	W/M	W/F	W/F	W/F	W/F	W/F	W/M	W/M	O/F
WBC ($\times 10^9$/L)	27.8	28.5	18.9	22.5	41.4	153.2	345.0	31.0	154.8	3.6	60.7	98.8	43.7	1.8	6.5	94.6	5.4	122.0
Hb (g/dL)	11.7	8.8	8.2	5.2	6.5	6.8	6.0	5.8	6.6	9.8	11.5	8.4	9.0	8.9	8.0	6.1	11.8	4.1
Platelets ($\times 10^9$/L)	10.0	3.0	7.0	82.0	75.0	7.0	110.0	131.0	8.0	209.0	159.0	65.0	66.0	20.0	39.0	14.0	437.0	9.0
FAB type	L-2	L-1	L-1	L-1	L-1	L-1	L-2	L-1	L-1	L-1	L-1	L-1	L-1	L-1	L-1	L-1	L-1	L-1
TdT	98	91	ND	99	94	98	84	94	94	88	7	0	86	36	72	80	0	91
E rosettes																		
37 °	1	6	7	1	1	1	0	0	4	0	3	4	0	0	0	1	1	0
4 °	1	1	0	0	0	0	0	ND	0	1	0	0	0	0	0	0	0	0
AET	2	10	8	3	11	5	1	6	3	2	6	10	5	1	3	1	5	15
T-101	1	9	3	4	9	2	2	10	4	10	11	12	4	7	5	4	11	ND
T-11	ND	ND	ND	3	12	5	2	6	6	7	6	13	6	6	2	6	8	ND
T-3	ND	ND	ND	5	17	4	4	5	0	10	6	9	3	8	5	5	8	ND
HLA-DR	100	73	55	73	89	49	86	84	84	88	74	76	88	96	74	90	89	ND
CALLA	46	88	77	99	90	1	0	96	92	74	87	2	85	91	80	82	96	ND
B4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	69	90	74	ND
p24	ND	ND	ND	100	73	80	73	90	88	12	75	63	91	90	80	85	94	ND
cIg	neg	92	neg	ND	67	2	48	80	1	0	0	0	90	0	0	0	85	0
sIg	3	7	3	0	25	2	0	0	2	1	1	1	1	0	0	1	4	5

Abbreviations: ND, not determined; AET, aminoethyl-isothiouronium bromide hydrobromide; neg, negative.
The findings are given as the percentage of positive cells unless otherwise noted.

having two rearranged κ-chain genes. The percentage of patients with rearranged light-chain genes is lower than that found in the studies of others[3] but is similar to the overall percentage of patients with light-chain gene rearrangements (8/39, 21%) that we have studied.

Serial studies in patient 1. Patient 1 was evaluated at diagnosis and again at relapse approximately 2 years later. The leukemic blasts were CALLA$^+$ at diagnosis but were CALLA$^-$ at relapse. Karyotypic analysis at diagnosis dem-

Table 3. Cytogenetic Findings

Patient No.	Karyotype
1D	45,XX,−20,−22,t(2;16)(p12;p11),+mar
1R	46,XX,t(4;11)(q21;q23)
2	46,XX,22q−/48,XX,22q−,+22q−,+mar
3	51,XY,+X,+6,+**14,**+17,+18,dup(1)(q21q32)
4	55,XX,+5,+6,+8,+18,+19,+20,+21,+22,+inv(1)(p12p34)
5	46,XY,t(9;12)(p22;p12)
6	46,XX,t(1;11)(p?32;q?23)
7	46,XY,t(4;11)(q21;q23)
8	54,XX,+4,+6,+10,+**14,**+17,+18,+21,+22
9	46,XX,del(9)(p21),del(12)(p12)
10	47,XY,+7
11	47,XX,+21
12	46,XX,t(10;11)(p13;q13)
13	54,XX,+X,+6,+10,+**14,**+18,+20,+21,+21
14	58,XX,+X,+4,+5,+6,+7,+10,+**14,**+**14,**+17,+18,+19,+21
15	57,XX,+5,+7,+**14,**+15,+17,+18,+21,+21,+del(1)(p32),+del(8)(p22),+i(9q)
16	52,XY,+6,+**14,**+17,+18,+22,+mar
17	55,XY,+X,+4,+5,+6,+9,+**14,**+18,+21,+21,i(17q)
18	57,XX,+X,+4,+6,+6,+8,+11,+**14,**+17,+18,+21,+21,dic(1)(q22−p21::q22−pter)

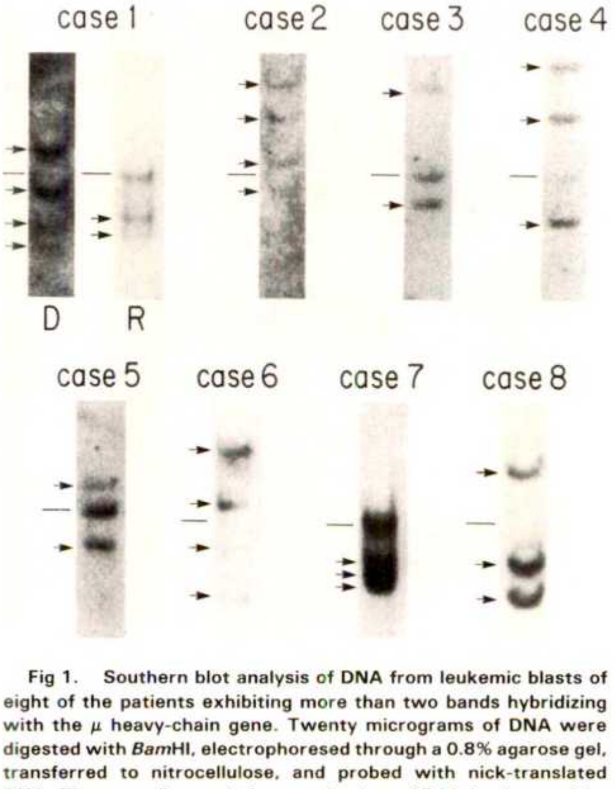

Fig 1. Southern blot analysis of DNA from leukemic blasts of eight of the patients exhibiting more than two bands hybridizing with the μ heavy-chain gene. Twenty micrograms of DNA were digested with *Bam*HI, electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and probed with nick-translated DNA. The germ line μ-chain gene is about 17 kb in size, and its location is indicated by the line. Rearranged bands for each case are indicated by the arrows. Abbreviations: D, diagnosis; R, relapse.

Table 4. Summary of Immunoglobulin Gene Rearrangement Data for the Patients With Greater Than Two μ-Gene Bands

Patient	Enzyme	Probe	μ Gene*	Enzyme	Probe	κ Gene
1 Dx	*Bam*HI	$C\mu$	4^+			ND
1 Rel	*Bam*HI	$C\mu$	2^+1°			ND
	*Hin*dIII	J_H				
2 Dx	*Bam*HI	$C\mu$	4^+			ND
3 Dx	*Bam*HI	$C\mu$	2^+1°			ND
4 Dx	*Bam*HI	$C\mu$	3^+	*Bam*HI	C_κ	1°
5 Dx	*Bam*HI	$C\mu$	2^+1°	*Bam*HI	C_κ	1°
6 Dx	*Bam*HI	$C\mu$	4^+			ND
7 Dx	*Bam*HI	$C\mu$	3^+1°	*Bam*HI	C_κ	1°
	*Hin*dIII	J_H				
8 Dx	*Bam*HI	$C\mu$	3^+	*Bam*HI	C_κ	1°
9 Dx	*Bam*HI	$C\mu$	3^+1°	*Bam*HI	C_κ	1°
	*Hin*dIII	J_H				
	*Eco*RI	$C\mu$				
10 Dx	*Bam*HI	$C\mu$	2^+1°	*Bam*HI	C_κ	1°
11 Dx	*Bam*HI	$C\mu$	3^+1°	*Bam*HI	C_κ	2^+
	*Hin*dIII	J_H				
12 Dx	*Bam*HI	$C\mu$	2^+1°	*Bam*HI	C_κ	2^+
	*Hin*dIII	J_H				
13 Dx	*Bam*HI	$C\mu$	2^+1°	*Bam*HI	C_κ	1°
	*Hin*dIII	J_H				
14 Dx	*Bam*HI	$C\mu$	2^+1°	*Bam*HI	C_κ	1°
	*Hin*dIII	J_H				
	*Eco*RI	$C\mu$				
15 Dx	*Bam*HI	$C\mu$	2^+1°	*Bam*HI	C_κ	1°
16 Dx	*Bam*HI	$C\mu$	4^+			ND
	*Hin*dIII	J_H				
	*Eco*RI	$C\mu$				
17 Dx	*Bam*HI	$C\mu$	3^+1°			ND
18 Dx	*Bam*HI	$C\mu$	3^+1°			ND
	*Hin*dIII	J_H				
	*Eco*RI	$C\mu$				

Abbreviations· Dx, diagnosis, Rel, relapse; $^+$, rearranged allele; $^\circ$, germ line allele, C_κ, κ light-chain constant-region probe.

*The number of heavy-chain genes indicated is based on the *Bam*HI probing and was confirmed by use of the other enzyme and probe combinations listed.

onstrated only one neoplastic clone with a t(2;16) translocation However, at relapse the t(2;16) abnormality could not be found, and a clone with the t(4;11) translocation was present (Table 3). Extensive analysis of metaphases from diagnosis did not demonstrate the presence of the t(4;11).

At diagnosis, four $C\mu$-hybridizing bands were present, all rearranged, but at relapse only two rearranged and one germ line band were present (Fig 1). The germ line band present at relapse probably resulted from the normal cells (30%) that were in the preparation used for DNA extraction. Since no DNA remained from diagnosis, we were unable to directly compare the sizes of the μ genes in both samples. However, the similarity in size of two of the bands from diagnosis (the bottom two bands) with the two non–germ line bands present at relapse suggests conservation (persistence) of the immunoglobulin μ-gene rearrangements from one clone.

DISCUSSION

Our findings confirm that in B precursor cell ALL at least one copy of the immunoglobulin μ heavy-chain gene is always rearranged.[3,9,28,29] The differentiation stage of our patient population appears earlier than other studies since only 21% had rearranged κ light-chain genes, compared with about 40%.[3] Because of the large percentage of early B precursor ALLs without light-chain gene rearrangement and the finding that μ heavy-chain gene rearrangements occur in about 20% of non–B cell leukemias,[4-6,30,31] immunoglobulin gene rearrangement data by itself cannot always be used as a definitive marker of B cell lineage. This data must be correlated with immunologic data to establish the B precursor cell origin of ALL.

An unexpected finding of this study was that DNA from 18 of 93 (19.4%) patients had more than two bands that hybridized to the $C\mu$ probe. This result was surprising because in the extensive studies of Korsmeyer et al[3,13,28] no cases with more than two μ-gene–hybridizing bands were reported. To rule out trivial explanations such as incomplete enzyme digestion or allelic variation in the *Bam*HI sites, we used several different combinations of restriction enzymes (*Eco*RI and *Hin*dIII) and μ-gene probes ($C\mu$ or J_H). For the nine patients analyzed by *Hin*dIII digestion and J_H probing and the four analyzed by *Eco*RI digestion and J_H probing, the results were entirely consistent with the *Bam*HI digestion and $C\mu$ probing, confirming the presence of more than two hybridizing bands. Data similar to ours has been noted by Foa et al[32] who found that five of 19 (26%) patients examined had three bands hybridizing to a J_H probe. These authors used both *Eco*RI and *Sac*I digests to confirm their findings Thus, in two independent studies, between 19% to 26% of the patients examined had more than two μ-gene–hybridizing genes in their leukemic clones at diagnosis. We do not know the reason(s) why Korsmeyer et al[3] did not detect such patients. Possibly there could be fundamental clinical and/or biologic differences in the patient populations studied by us, Foa et al (patient's ages were not given), and that studied by Korsmeyer et al.

We considered two alternative explanations for the presence of the extra bands: (1) It is possible that extra copies of chromosome 14 were present and the rearrangements of the μ heavy-chain gene on the duplicated chromosome differs from that of the parent chromosome. (2) Two populations of cells were present, either related by clonal evolution, or unrelated and the product of two separate transformation events (biclonal).

Detailed examination of the karyotypes of the 18 patients demonstrated that eight had extra copies of chromosome 14 (Table 3); this is a known characteristic of the greater-than-50 ploidy group.[33] However, in only four patients (nos. 3, 8, 13, and 15) did the number of copies of chromosome 14 agree with the number of bands seen on Southern blots. Of the remaining four patients, one (no. 14) had four copies of chromosome 14 but only three μ-gene–hybridizing bands. Analysis of the gel from this patient revealed that hybridization to one of the three bands was greater than that to the other two, suggesting that the same gene rearrangement may be present on two copies of chromosome 14 (data not shown). Three patients (nos. 16 to 18) had four copies of the μ gene but only three copies of chromosome 14. In two of these patients (nos. 17 and 18), germ line μ-gene bands were present, and although differential blast counts were over 90% for these two patients, it is possible that sufficient normal cells were present to allow us to detect the germ line μ gene. Thus, except for the remaining patient with three copies of chromosome 14 and four Cμ-hybridizing bands, the data indicate that the extra copy or copies of chromosome 14 is responsible for the presence of more than two bands that hybridize with the μ-chain gene probes in this group of patients. These results also suggest that the rearrangement of the μ heavy-chain gene can occur after the occurrence of chromosomal duplication.

Although extra copies of chromosome 14 may be an explanation for the presence of extra μ-gene–hybridizing bands in some patients, in the majority of patients only the normal number of copies of chromosome 14 were present. In these cases, the data lead us to conclude that more than one population of cells is present. The second population could be the result of either separate leukemic transformation events, ie, biclonal leukemia, or could represent sublines that have evolved from the original clone. There is precedent for both of these possibilities.

Biclonal B cell lymphomas have been identified by Sklar et al.[34] Each biclonal tumor contained cells bearing individually distinctive idiotypes. The presence of each idiotype on different cells was confirmed by cell sorting and the contribution of each cell population to the μ heavy-chain gene pattern on Southern blots determined. Such cell-sorting experiments are not possible with the types of leukemias studied here since all are very early B cell precursors, none had sIg, and most do not even express cytoplasmic immunoglobulin (Table 2). Independently arising clones would be expected, however, to have different cytogenetic abnormalities, and two distinct stem lines were not found in the karyotypes of any of the patients (Table 3). We do not therefore believe that biclonality is the explanation for our findings of more than two Cμ-hybridizing bands in the ten patients without extra copies of chromsome 14.

The more likely explanation is clonal evolution involving continued rearrangement of the Ig genes after leukemic transformation. Growth of clonal lines of Abelson murine leukemia virus (A-MuLV)-transformed null (pre-B) mouse lymphoid cells is accompanied by continued rearrangement of the heavy-chain genes.[35] Alt et al[35] postulate that the initial Ig gene rearrangements involved D_H-J_H joinings, which could be rearranged further by the substitution of another D_H segment or by the addition of a V_H segment to the existing D_H-J_H complex These results have recently been confirmed and extended by Whitlock et al[36] who showed that A-MuLV transformants of a cloned mouse pre–B cell population exhibited multiple heavy- and light-chain Ig gene rearrangements. Thus, in the murine lymphoid leukemic cell counterparts to human pre-B lymphoid leukemias, transformation does not preclude further rearrangements in the Ig gene loci. If this mechanism is responsible for the generation of the two cell populations in the human leukemias we have examined, proof could come from cloning the genes and examining the V_H-D_H-J_H junctions. Such studies are in progress.

Although all patients received intensive multiple-agent chemotherapy,[16–18] eight of the 18 with more than two Cμ-gene bands have relapsed. Interestingly, there appears to be a direct correlation between the treatment outcome and karyotype in this group of patients. Seven of the eight patients with an extra chromosome 14 remain in continuous complete remission with a median follow-up of 1.5+ years (range, 0.9 to 1.8+ years), consistent with our previous observations of the comparatively good prognosis of hyperdiploid ALL.[37] In contrast, of the ten patients without an extra chromosome 14, only three remain in complete continuous remission (1.8+, 2.0+, and 3.0+ years) with a median time to relapse of 0.95 years (range, 0.2 to 2.2 years) Although these results must be considered preliminary because of the number of patients and length of follow-up, they are consistent with clonal evolution and further somatic mutation as a mechanism underlying leukemic progression and acquisition and emergence of drug resistance.[38–40]

In summary, we have found that around 20% of childhood ALLs at diagnosis have more than two Cμ-hybridizing bands, which can be the result of either extra copies of chromosome 14 or of clonal evolution. The presence of extra Cμ-hybridizing bands without an extra chromosome 14 seems to be correlated with a risk of early relapse.

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Lymphocyte Malignancy and Chromosome 14: Structural Aberrations Involving Band q11

By Nanette Clare, David Boldt, Gerald Messerschmidt, Paul Zeltzer, Kathryn Hansen, and Louis Manhoff

Five patients with lymphocytic malignancies were found to have structural aberrations of chromosome 14, all involving band q11. The malignant cells of all five cases were analyzed by immunofluorescence to establish immunologic phenotypes. Three patients had T lymphoblastic lymphoma/leukemia with mediastinal masses (cases 1, 2, and 3); one patient had peripheral T cell lymphoma (case 4); and one patient had acute lymphocytic leukemia, common acute lymphoblastic leukemia antigen positive (case 5). Although aberrations of chromosome 14 frequently are associated with lymphocytic malignancies, these abnormalities most often result in extra material on the long arm, 14q+. However, none of the five patients reported here had a 14q+. Cases 1 and 2 had t(9;14)(q34;q11) and t(11;14)(p11or13;q11), respectively. Case 3 showed del(14)(q11), and cases 4 and 5 showed inv(14)(q11q32). Structural aberrations resulting in 14q− and inv(14) appear to occur infrequently. The five cases in this report, in conjunction with those found in the literature, indicate a strong association between breaks at 14q11 and T lymphocytic malignancies.

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CYTOGENETIC analyses of lymphocytic malignancies are being reported with increasing frequency. Structural abnormalities of chromosome 14 have been commonly found, particularly the t(8;14)(q24;q32) seen in Burkitt's lymphoma and other types of B lymphocytic malignancies.[1-3] Most structural aberrations of chromosome 14 associated with lymphocytic disorders result from translocations of significant portions of other chromosomes to chromosome 14, showing 14q+ with the break point at band 14q32, the location of the heavy-chain gene for immunoglobulin. Only rarely is there a major loss or translocation of no. 14 resulting in a 14q−.

Chromosome analysis of the lymphomas has not progressed as rapidly as that in leukemia, principally because of the technical problems of obtaining sufficient numbers of cells in mitosis from solid tissue There have been few reports of chromosome findings in T cell disorders, most occurring in T-chronic lymphocytic leukemia (T-CLL) or cutaneous T cell lymphoma (CTCL). Some authors report minimal or no involvement of no. 14,[4-6] whereas more recent studies have shown structural aberrations involving band q11. These have consisted either of an inversion, inv(14)(q11q32), or translocation resulting in 14q−[7-10] These are new and unusual chromosome aberrations of lymphocytic malignancies that occur principally in T cell disorders.

Reported here are five cases of lymphocytic malignancies with abnormalities involving 14q11. Four of these cases were classified as T cell malignancies and one as acute lymphocytic leukemia, pre–B cell type.

CASE HISTORIES

Case histories and pertinent data are summarized in Table 1

MATERIALS AND METHODS

Bone marrow aspirations and biopsies were performed on each patient after informed consent

Cell surface markers Cell suspensions prepared from bone marrow or peripheral blood by density sedimentation on Ficoll-Hypaque[13] were examined for reactivity with panels of commercially available, well-characterized mouse monoclonal antibodies to human lymphocyte antigens. Monoclonal antibodies and their specificities were as follows: T11, total T cells and thymocytes; OKT3 and anti–leu-1, mature T cells and mature thymocytes; OKT4, T-helper cells and mature thymocytes; OKT6, cortical thymocytes; OKT8, T-suppressor cells and mature thymocytes, OKT9 and OKT10, early thymocytes and other hematopoietic cell precursors, J5, anti–common acute lymphoblastic leukemia antigen (CALLA) on leukemia cells and normal lymphoid precursor cells; B1 and B4, total B cells; I2, anti-Ia antigen Monoclonal reagent anti–leu-1 was obtained from Becton-Dickinson Monoclonal Antibody Center, Sunnyvale, Calif; T11, I2, B1, B4, and J5 from Coulter Immunology, Hialeah, Fla; and all others from Ortho Diagnostics, Westwood, Mass

Expression of T and B lymphocyte surface markers by malignant cells was assessed by indirect immunofluorescence and flow cytometry[14] Briefly, 1 to 2 × 10^6 cells were incubated for 30 minutes at 4 °C with an appropriate dilution of monoclonal antibody. Cells were washed twice, then incubated for an additional 30 minutes at 4 °C with a 1:50 dilution of fluorescein-conjugated goat antimouse IgG. After two additional washes, cells were suspended in 1% buffered paraformaldehyde, then analyzed by flow cytometry on a Coulter TPS 1 fluorescence-activated cell sorter with a 2W laser. (Patient 2 was analyzed on an Ortho Cytofluorograph) In parallel incubation to control for nonspecific binding, cells were incubated with mouse ascites fluid containing nonrelevant immunoglobulin in place of specific monoclonal antibody during the first step, but otherwise were treated identically

Cytochemistry. Cytochemistry techniques used to analyze malignant cells were those in routine use in our clinical laboratories.[15] They include myeloperoxidase, Sudan black B, ASD-chloroacetate esterase, alpha napthyl butyrate esterase with and without sodium fluoride, periodic-acid Schiff (PAS), and acid phosphatase. Terminal deoxynucleotidyl transferase (TdT) was assayed on fixed smears by an immunofluorescent method.[16]

Cytogenetics Initial specimens were received from each patient at the time of diagnosis, prior to therapy. Peripheral blood, 1 to 10 mL, or 0.5 mL bone marrow (patient 3) was collected in EDTA anticoagulant[17] and kept at 4 °C until received in the cytogenetic laboratory the same day There was not sufficient bone marrow aspirate from patients 1, 2, 4, or 5 for cytogenetic analysis The specimens were processed by a modification of the standard direct harvest procedure,[18] unstimulated 24-hour culture, and/or by incu-

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Table 1. Summary of Clinical Data

Case No	Age (yr/sex)	Diagnosis	Presenting Signs	Percentage of WBC*/Blasts	Percentage of Marrow Blasts	Therapy	Clinical Course
1	23 male	T cell lymphoblastic lymphoma/leukemia	fever mediastinal mass adenopathy superior vena cava syndrome CSF, pleural fluid blasts	21,000 84%	100	modified L10M[11] mediastinal and CNS radiation	remission lost to follow-up
2	29 female	T cell lymphoblastic lymphoma/leukemia	mediastinal mass mandibular abscess	94,900 94%	95	cyclophosphamide vincristine Adriamycin L-asparaginase methotrexate prednisone[12]	remission at 4 mo
3.	4½ male	T cell lymphoblastic lymphoma/leukemia	mediastinal mass adenopathy CSF blasts	35,500 17%	38	prednisone, vincristine daunorubicin L-asparaginase CNS cytosine arabinoside and methotrexate	remission at 3 mo
4.	60 male	peripheral T cell lymphoma	adenopathy skin, duodenum, and CSF involvement	15,000 92%	100	cyclophosphamide Adriamycin, vincristine prednisone CNS methotrexate	expired 26 d postdiagnosis
5	22 male	pre-B ALL	adenopathy splenomegaly hepatomegaly	171,000 95%	90	modified L10M protocol[11]	remission at 9 mo

Abbreviation: ALL, acute lymphocytic leukemia.

*Peripheral WBC count per μL

bation in media with phytohemagglutinin for 48 to 96 hours [19] Slides were stained with trypsin-Giemsa for G-bands.[20] Additional bone marrow and peripheral blood specimens were obtained from patients 2 and 5 during complete remission. The bone marrow (1 mL) was received in EDTA and processed by direct harvest. The peripheral blood (10 mL) was received in sodium heparin and processed by a 72-hour incubation with phytohemagglutinin Slides from both samples were G-banded as previously.

RESULTS

Cell surface markers. Results of surface marker studies and cytochemistry are given in Table 2.

Cytogenetics. The cytogenetic results are summarized in Fig 1 and Table 3.

The majority of cells analyzed in cases 1 and 4 were obtained from the 72-hour phytohemagglutinin-stimulated cultures. In case 1, five of eight cells present in the direct harvest were found to have the abnormal karyotype. The chromosome 9 involved in the translocation with the 14 also showed a terminal deletion of the short arm at p21 as the only other aberration present. Case 4 contained t(9;?) (q34;?) and t(13;?) (p12?;?) in addition to the inv(14)(q11q32). The origin of the 9q+ material was not determined; the t(13;?) was suggestive of a 13;21 translocation.

The cells analyzed in cases 2 and 3 were obtained by direct harvest. The banding was not of sufficient quality in case 2 to determine the exact break point in the 11, appearing to be at either p11 or p13. The 14q− is longer in this case than in cases 1 and 3 because of the translocated 11p material. The abnormality in case 3 may actually represent a 7;14 translocation; however, this could not be identified with certainty. The majority of cells analyzed in case 5 were obtained by 24-hour unstimulated culture.

DISCUSSION

Five cases of lymphocytic malignancies are reported with chromosome aberrations involving band 14q11. In two cases the abnormality consisted of a translocation, in one case a deletion, and in two cases a paracentric inversion. In none of the cases did the abnormality result in 14q+.

Lymphocyte surface marker data were available for all five patients. Abnormal lymphocytes marked as T cells in four patients (cases 1, 2, 3, and 4) and as pre–B cells in one patient (case 5). Cells from patients 3 and 4 had similar phenotypes. These cells expressed the E rosette receptor as detected by reactivity by monoclonal reagents T11 and anti–leu-5. In case 4 the cells also expressed the T3 antigen, and in cases 3 and 4 both helper (T4/leu-3)- and suppressor (T8/leu-2a)-specific antigens were present. The phenotypes were T11+T3+T4+T8+CALLA-Ia-Bl-. This phenotype is most consistent with a level of maturation at the stage of the common thymocyte.[21] The T10 positivity observed in case 4 further supports a thymocyte origin.

Less extensive data were available for case 2, but the TdT positivity together with high levels of expression of T11, leu-1, and T3 is most consistent with a thymocyte level of maturation Case 1 was characterized by relatively low levels of reactivity with all monoclonal reagents. Evidence supporting a T cell derivation includes expression of the T11, T6, and T10 antigens as well as polar acid phosphatase positivity. Expression of T6 and T10 but not T3, T4, or T8 places the

Table 2. Morphologic, Cytochemical, and Surface Marker Characteristics of Malignant Lymphocytes

			Surface Markers	
Case	Morphology	Cytochemistry	T Cell (Percentage positive)	B Cell
1	convoluted nuclei with diffuse, immature chromatin	TdT-, PAS-, and acid phosphatase-positive, peroxidase-, Sudan black-, and esterases-negative	T11 (19)	CALLA (12)
			leu-1 ND	B1 (2)
			T3 (1)	Ia (10)
			T4 (0)	
			T6 (17)	
			T8 (0)	
			T9 ND	
			T10 (15)	
2	convoluted nuclei with diffuse, immature chromatin	TdT-positive	T11 (99)	CALLA (0)
			leu-1 (71)	B1,Ia ND
			T3 (25)	
			T4,T6,T8,T9,T10 ND	
3	tightly convoluted nuclei with diffuse, immature chromatin	TdT-negative, Sudan black-, nonspecific esterase-negative; acid phosphatase-positive	T11 (62)	CALLA (8)
			leu-5 (72)	B1 (0)
			T8 (81)	SMIg (3)
			leu-2a (68)	
			T4 (63)	
			leu-3 (70)	
			T3,T6,T9,T10 ND	
4	convoluted nuclei with irregularly clumped chromatin	cytochemistries ND	T11 (65)	CALLA (0)
			leu-1 ND	B1 (2)
			T3 (76)	Ia (4)
			T4 (59)	
			T6 ND	
			T8 (40)	
			T9 (2)	
			T10 (35)	
5	irregular nuclei with diffuse, immature chromatin	TdT-positive PAS-, peroxidase-, Sudan black-, esterases-negative	T11 (3)	CALLA (95)
			other T markers ND	B1 (3)
				B4 (97)
				Ia (78)

Abbreviations ND, not determined, SMIg, surface membrane immunoglobulin.

level of maturation at an early thymocyte stage. In case 1, 12% of the lymphoblasts also expressed CALLA. CALLA-positive T cell malignancies have been previously recognized[22–24] The phenotype of case 5 was CALLA-positive, TdT-positive, IA-positive, and B4-positive. This is characteristic for pre-B ALL.[21]

T cell disorders have been, until recently, reported only rarely in this country. This is most likely due to inexperience in detecting T cell malignancies histologically and to a lack of obtaining marker studies in diffuse lymphomas. It is likely that T cell malignancies are more prevalent than originally thought. The majority of cytogenetic analyses of T cell malignancies reported are T-CLL and CTCL.[7–9,25–32] Classification of T cell malignancies is in the formative stages, and as marker studies become standard procedures, an improved classification may result. With the morphology, cytochemistry, and surface marker data, the cases reported here were classified as T cell lymphoblastic lymphoma/leukemia (cases 1 to 3), peripheral T cell lymphoma (case 4), and ALL, pre-B type (case 5).

The majority of lymphocytic malignancies with chromosome aberrations reported in the literature to date involve structural and/or numerical aberrations of chromosome 14. Most of these structural aberrations involve the terminal band on the long arm, 14q32, and consist of a translocation, 14q+, with chromosome 14 being the recipient of a major portion of another chromosome. This type of abnormality was described originally in Burkitt's lymphoma,[33] but it has been seen also in lymphocytic malignancies. Although t(8;14) is most common, t(2;8) or t(8;22) have also been reported.[2,3] These observations had led to speculation that the 8q− may be more significant in the development of malignancy than the 14q+.[1] However, it has recently been found that the *c-myc* oncogene, located at 8q24, is deregulated when involved in the translocations.[2] In the t(8;14) the break point in no. 8 is directly proximal to the *c-myc* locus so that it is translocated to the 14q32 at various sites of the heavy-chain locus, frequently involving the μ-chain switch region. The t(2;8) and t(8;22), on the other hand, involve a break point on no. 8 just distal to the *c-myc* locus so that the λ-chain locus from no. 22 and the κ-chain locus from no. 2 are attached to that site.[2] These rearrangements most often involve B cell malignancies.

Cytogenetic analysis is proving to be important in the accurate diagnosis and possibly in the prognosis of lymphocytic lymphomas,[34,35] and there are two reports that suggest it may have predictive value. McCaw et al[10] studied T lymphocytes in ataxia telangiectasia and found five cases showing

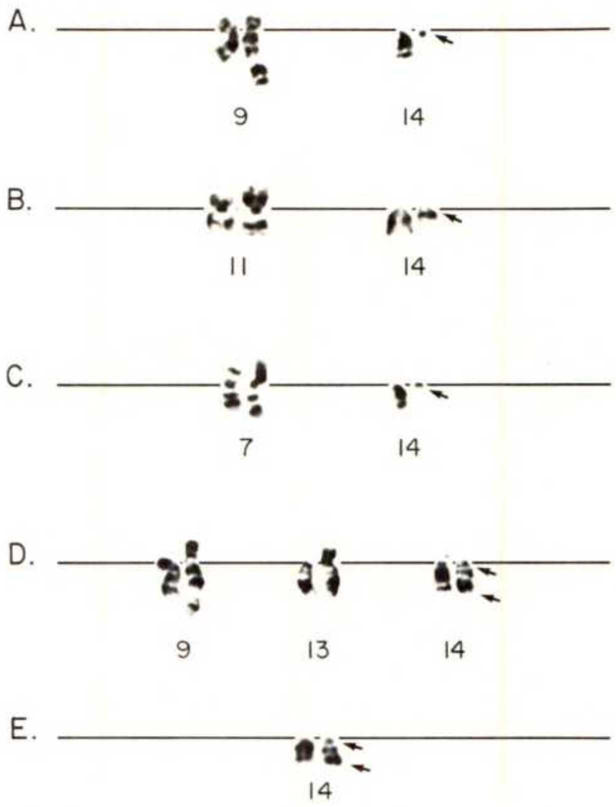

Fig 1. Partial karyotypes of patients 1 to 5, A to E, respectively, showing chromosome aberrations (G-band with trypsin-Giemsa). (A) del(9)(p21),t(9;14)(q34;q11). (B) t(11;14)(p11or13;q11). (C) 7p+,del(14)(q11). (D) t(9;?)(q34;?),t(13;?)(p12?;?),inv(14)(q11q32) (E) inv(14)(q11q32).

14q– or Dq– that were very similar in appearance to a break at 14q11 (these were not included in Fig 2). Chronic lymphocytic leukemia that involved the clone with t(14q–;14q+) developed in one of these patients. Fukuhara et al[36] demonstrated clonal aberrations in stimulated peripheral lymphocytes of two healthy adult carriers of adult T cell leukemia–associated antigen; these may prove to be markers of a premalignant clone.

Chromosome 6 has also been implicated as being frequently involved in numerical or structural aberrations of T cell disorders. Miyamoto et al[39] reported 17 of 30 patients with either trisomy 6 or 6q–, and Whang-Peng et al[40] found 6q deletions in six of 11 patients with T cell leukemia/lymphoma. These six patients shared several clinical findings and had a significantly shorter survival than patients without 6q–. This may indicate prognostic significance of chromosome 6 abnormalities in T cell disorders.

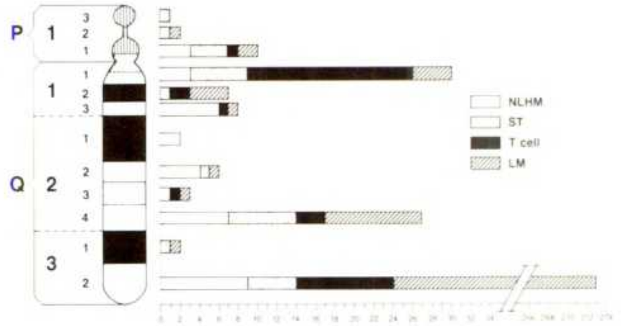

Fig 2. Diagram of chromosome 14 showing the number of different malignant disorders involving aberrations of the various bands. From references 7–9, 25–32, 36–38 and present cases. Abbreviations: NLHM, nonlymphocytic hematopoietic malignancy; ST, solid tumor; T cell, T cell lymphocytic malignancies; LM, lymphocytic malignancies, non–T cell.

Figure 2 is a diagram of chromosome 14 showing the relative incidence of aberrations involving the various bands of the chromosome. There is difficulty in constructing this kind of chart since many reports do not include surface marker studies. For the purpose of simplicity, all B cell disorders and some of those that were not differentiated were included in the category of lymphocytic malignancy (LM). Only those cases proven to be T cell malignancies by marker studies and/or cytochemistry were placed in the category of T cell. The diagram emphasizes the overwhelming number of cases involving band q32. Of note, only 3.6% (10/273) of these cases can be shown conclusively to be T cell disorders.[30-32,37] The T cell disorders listed with aberrations involving 14q32 include only those with a single break point. If the cases with two break points involved are included (q11 and q32),[7-9,32] then 7% (20/284) of cases affecting 14q32 are T cell disorders. Case 5 of this report would be included as LM at q32. The band of chromosome 14 involved next most commonly with malignancies is q11. Of all malignancies associated with the q11, 56.6% (17/30) can be classified as T

Table 3. Cytogenetic Analysis, Trypsin-Giemsa Banding

Case No.	Specimen	No. Cells Counted/Analyzed	Karyotype
1	PB, pretreatment	54/44	46,XY,del(9)(p21),t(9;14)(q34;q11)—45% 46,XY—55%
2	PB, pretreatment	39/28	46,XX,t(11;14)(p11 or 13;q11)
	BM, remission	37/25	46,XX
3	BM, pretreatment	53/42	46,XY,7p+,del(14)(q11)—24% 46,XY,—76%
4	PB, pretreatment	34/20	46,XY,t(9;?)(q34;?),t(13;?)(p12?;?), inv(14)(q11q32)
5	PB, pretreatment	32/22	46,XY,inv(14)(q11q32)
	PB, remission	35/23	46,XY
	BM, remission	30/25	46,XY

Abbreviations: PB, peripheral blood; BM, bone marrow.

cell in origin. If the LM are considered separately, T cell disorders account for 81% (17/21). Therefore, whereas band 14q32 is strongly associated with B lymphocyte malignancies, band 14q11 appears to be associated with T cell malignancies.

A recently published report by Hecht et al[41] also shows this association, with three cases of T cell malignancies having breaks at 14q11. The authors also state that rearrangements of chromosome 14 that result in approximation of 14q32 to 14q11 have only occurred in T cell malignancies. Case 5 in this report, however, has inv(14) (q11q32) and is a pre-B ALL.

Analysis of the T cell disorders involving clonal aberrations of 14q11[7,8,9,32,38] shows eight of 17 to be inv(14) (q11q32), eight of 17 to involve a translocation with chromosomes 9 (three cases), 14 (two cases), 4, 11, and 17 (one case each), and one of 17 to involve a deletion that may actually be a t(7;14). There is no preferential involvement of any other chromosomes with these 14q11 abnormalities. The importance of band 14q32 in T cell disorders cannot be dismissed, since ten of 17 cases involve breaks of both 14q11 and q32 [t(14;14) and inv(14)]. Recent reports[7,8,9,35] have also stressed the association of T cell disorders and aberrations of 14q11; these cases have been predominantly T-CLL and CTCL. The present cases show that this aberration may occur in other types of T cell disorders as well. It is important and interesting to note that Croce et al[42] have most recently mapped the gene for the α chain of the T cell receptor to chromosome 14 in the region q11-q12. A break in this region likely results in a different environment for this gene, analogous to translocations in B cell malignancies involving the immunoglobulin heavy-chain and light-chain loci. Further studies may also implicate oncogenes in these aberrations, as speculated by Croce et al.[42]

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Calcium Accumulated by Sickle Cell Anemia Red Cells Does Not Affect Their Potassium ($^{86}Rb^+$) Flux Components

By Olga E. Ortiz, Virgilio L. Lew, and Robert M. Bookchin

We investigate here the hypothesis that the high Ca content of sickle cell anemia (SS) red cells may produce a sustained activation of the Ca^{2+}-dependent K^+ permeability (Gardos effect) and that the particularly high Ca levels in the dense SS cell fraction rich in irreversibly sickled cells (ISCs) might account for the Na pump inhibition observed in these cells. We measured active and passive $^{86}Rb^+$ influx (as a marker for K^+) in density-fractionated SS cells before and after extraction of their excess Ca by exposure to the Ca ionophore (A23187) and ethylene glycol tetra-acetic acid and with or without adenosine triphosphate depletion or addition of quinine. None of these maneuvers revealed any evidence of a Ca^{2+}-dependent K leak in SS discocytes or dense cells. Na pump inhibition in the dense SS cells was associated with normal activation by external K^+ and a low V_{max} that persisted after Ca extraction from the cells. These results are consistent with our recent findings that the excess Ca in these cells is compartmentalized in intracellular inside-out vesicles and unavailable as free Ca^{2+} to the inner membrane surface. Although the steady-state free cytoplasmic Ca^{2+} in oxygenated SS cells must be below the levels needed to activate the K^+ channel, possible brief activation of the channels of some SS cells resulting from transient elevations of cell Ca^{2+} during deoxygenation-induced sickling cannot be excluded. The dense, ISC-rich SS cell fraction showed a Ca^{2+}-independent increase in the ouabain-resistant, nonsaturable component of $^{86}Rb^+$ influx that, if uncompensated by Na^+ gain, could contribute to the dehydration of these cells.
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BECAUSE of the high-power–concentration dependence of hemoglobin (Hb) S polymerization,[1] the mechanism by which a substantial proportion of sickle cell anemia (SS) red cells become dehydrated has been the subject of intense scrutiny. A candidate often implicated in the dehydration has been the Ca^{2+}-sensitive K^+-selective channel[2,3] since, in principle, the documented high Ca content of SS cells could activate the channel, causing net KCl loss and cell shrinkage. Although measurements of $^{42}K^+$ fluxes in fresh, substrate-fed SS red cells in the presence and absence of quinine have ruled out any large increase in Ca^{2+}-dependent K^+ fluxes,[4] possible minor, sustained increases in K^+ flux, particularly in the dense SS cell fraction rich in irreversibly sickled cells (ISCs) in which the highest Ca levels are found,[5,6] have not yet been excluded. If present, such an uncompensated K^+ efflux could lead to slow cumulative shrinkage of the cells.

It has also been suggested that the high Ca content of ISCs might account for the Na pump inhibition observed in this SS cell fraction.[7] Such an abnormality could not itself account for cell dehydration but should be detectable if a substantial fraction of the total Ca content of ISCs were present as free ionized Ca in the cytoplasm.[8]

To assess the possible role of endogenous SS cell Ca in active and passive K^+ fluxes, we measured the influx of $^{86}Rb^+$ as a marker for K^+ in density-fractionated SS cells before and after extracting all their mobilizable Ca by exposure to ionophore and ethylene glycol tetra-acetic acid (EGTA) under a variety of conditions designed to reveal K^+-channel activity. The results confirmed Na pump inhibition in the ISC fraction and showed evidence of an increase in nonsaturable ouabain-resistant $^{86}Rb^+$ influx in the same cells. But there was no evidence of endogenous Ca effects on either pump or leak in any SS cell fraction.

MATERIALS AND METHODS

Heparinized venous blood was obtained with informed consent from normal donors and from patients with SS whose genotype has been established by electrophoretic and family studies at our Heredity Clinic The blood was kept refrigerated at 4 °C, and all experiments were performed within 24 hours of venesection The plasma and buffy coat were removed by aspiration after centrifugation, and the RBCs were routinely washed four times (unless stated otherwise) in the buffer appropriate to each experiment.

Density gradient separation of RBCs Density fractionation of the RBCs followed the method of Corash et al[9] with minor modifications. The RBCs were washed and suspended at a 20% hematocrit (HCT) value in buffered saline with potassium and glucose (BSKG) containing (in mmol/L) NaCl, 134, KCl, 5; glucose, 2; and Na-phosphate buffer, 10, pH 7.4 (37 °C), adjusted to 291 mosm with a Micro Osmometer, Model 5004 (Precision Systems, Sudbury, Mass)

Stractan (arabinogalactan, St Regis Paper Co, Libby, Mont) was dissolved in water and deionized by successive-batch equilibrations for several hours each at 4 °C with AG501-X8(D)20 50–mesh mixed-bed resin (Bio-Rad Laboratories, Richmond, Calif) until the pH had fallen to about 3.0 and the osmolarity to 100 mosm or less After the last filtration to remove the resin, the Stractan solution was centrifuged at 20,000 g for 45 minutes at 4 °C This stock solution, about 40% (wt/wt) Stractan, density 1.163 to 1.170, was diluted with BSKG to obtain four additional densities, 1.087, 1.095, 1 105, and 1 118, as measured on a refractometer (Model 33-45-58, Bausch and Lomb, Austin, Tex), centrifuged again for 30 minutes, passed through a 0.45-μm filter (type S, Nalgene, Rochester, NY), and stored frozen A discontinuous gradient was made by layering 2.5 mL of each of these four density solutions onto a cushion of 0.5 mL of the stock solution in 12-mL plastic centrifuge tubes; 1 mL of the 20% RBC suspension was layered onto each gradient tube, and they were centrifuged for 45 minutes at 17,000 rpm in an SS24 angle rotor of a Sorvall RC2B centrifuge (Ivan Sorvall, Newtown, Conn.) at 4 °C. The RBC bands were harvested with a Pasteur pipette and, after washing in the appropriate buffer, were sampled into buffered formalin for morphologic evaluation using a Zeiss microscope

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equipped with Nomarsky optics. Cells were designated ISCs if their length exceeded their width by a factor of two or more. Small crumpled RBCs seen in the dense fractions, which did not meet the criteria for ISCs, were designated deformed Cells with a density between 1 095 and 1.105 were 94% to 95% discocytes, with less than 1% reticulocytes seen on films stained with new methylene blue, and were used as the discocyte fraction; cells with a density between 1 118 and 1.163 were 68% to 73% ISCs, 18% to 19% deformed, and 11% to 14% discocytes and were used as the ISC-rich fraction.

Removal of endogenous RBC calcium. Cells were washed in buffer A containing (in mmol/L) NaCl, 75; KCl, 75; EGTA, 0 1, and Na-HEPES, 20, pH 7 6 (20 °C), 291 mosm. They were then suspended at a 30% HCT value in the same medium containing, in addition, 1 mmol/L EGTA, 0.2 mmol/L $MgCl_2$, and 10 μmol/L of the calcium inophore A23187 (Sigma Chemical Co, St Louis) and incubated at 37 °C for 15 minutes. The concentrations of Na^+, K^+, and Mg^{2+} in the medium were chosen to maintain cell concentrations of cations (other than Ca) in the presence of A23187 and EGTA in the Ca^{2+}-free medium.[10] Following incubation, the suspension was chilled on ice and diluted with 4 vol of buffer A containing 1 mmol/L EGTA, 10 mmol/L inosine, and 1% bovine serum albumin and washed twice with the same solution to remove all A23187 from the cell membranes and twice with the buffer appropriate to the experiments.

Depletion of RBC adenosine triphosphate The RBCs were washed in buffer A, resuspended at a 20% HCT value in the same medium with the addition of 10 mmol/L inosine and 6 mmol/L iodoacetamide,[11] incubated at 37 °C for 90 minutes, and then washed thrice in the appropriate buffer. The adenosine triphosphate (ATP) content of the RBCs, measured by the firefly technique,[12] was 0.8 to 1.0 mmol/340 g Hb in the fed cells, and after depletion, was less than 20 μmol/340 g Hb.

Measurements of $^{86}Rb^+$ influx in RBCs. For measurements of $^{86}Rb^+$ influx as a function of external K^+ concentration, illustrated in Fig 1, the procedure of Glynn[13] was used with modifications. Discocyte and ISC-rich SS cell fractions were washed in 4 vol of buffer B containing (in mmol/L) NaCl, 150; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 2; and Tris-phosphate, 2.5, pH 7.4 (37 °C) at 4 °C. The packed cells were resuspended at a 5% HCT value in cold incubation medium, and solid glucose and ouabain were added to give final concentrations of 10 mmol/L and 10^{-4} mmol/L, respectively When K^+ was present in the medium, the Na^+ concentration was (150 − K^+) mmol/L. $^{86}Rb^+$ was added as $^{86}RbCl$, 10 mCi/mg, (New England Nuclear, Boston) to provide a 10 μCi/mL suspension, and after taking zero-time samples at 0 °C, the suspensions were transfered to a 37 °C water bath with magnetic stirrers and sampled again at 20, 40, and 60 minutes. Samples of 100 μL of RBC suspension were delivered to 1.5 mL Eppendorf tubes in an ice bath containing 0 4 mL dibutyl phthalate (d^{20} = 1 047) (Matheson, Coleman and Bell, Norwood, Ohio) and 0.9 mL of K^+-free buffer B; the tubes were inverted and immediately spun for 10 seconds at 13,000 rpm in an Eppendorf microcentrifuge.[10] After aspiration of the supernatant, the tube walls were wiped with cotton swabs, the RBC pellets lysed by the addition of 0.1 mL of water containing 0.1% Triton X-100 (Sigma Chemical, St Louis), followed by 0.5 mL of 6% trichloracetic acid, and after centrifugation for one minute, 0.5 mL of clear supernatant was mixed with 5 mL Aquasol (New England Nuclear) for liquid scintillation counting. The specific activity of ^{86}Rb in each suspension was determined by processing aliquots of the whole suspension and referred to the K^+ concentration in the media. The Hb content of the cell suspensions was measured by the cyanomethemoglobin method, and the RBC $^{86}Rb^+$ uptake was expressed as mmol/340 g Hb.

The ouabain-sensitive component of the K^+ ($^{86}Rb^+$) influx was calculated by subtracting, at each time point, the $^{86}Rb^+$ uptake in the presence of 10^{-4} mol/L ouabain from the uptake by ouabain-free control suspensions and plotting the values as a function of the external K^+ concentration ($[K^+]_0$).

In all cases the ouabain-sensitive $^{86}Rb^+$ influx was linear with time for at least 40 minutes. At a $[K^+]_0$ higher than 2 mmol/L, on the other hand, the ouabain-resistant influx was high at 0 °C and leveled off with time at 37 °C, becoming linear between 20 and 60 minutes. Slopes were drawn by eye over the linear portions of these curves and plotted as a function of $[K^+]_0$. The apparent affinity and V_{max} of the ouabain-sensitive influx were computed from a fit to Hill's equation[14] with n = 2.

Specific variations in the suspension media and in sampling times for the experiments shown in Fig 2 and Tables 1 and 2 are described in their legends

Measurement of RBC cation contents The total RBC Ca was measured by atomic absorption spectroscopy as described previously.[15] For determination of cell [Na] and [K], the cells were washed four times in 20 vol of ice-cold buffer containing (in mmol/L) $MgCl_2$, 108, and Tris-Cl, 10, pH 7.4 (20 °C), 291 mosm, and lysed in a 15-mmol/L LiCl solution, and [Na] and [K] were measured by flame photometry (Model 143, Instrumentation Laboratories, Lexington, Mass). The Hb content of an aliquot of each lysate was measured and the results expressed as mmol/340 g Hb.

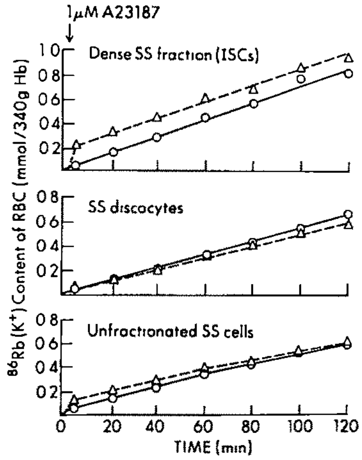

Fig 1. **Comparison of an active (ouabain-sensitive) and passive (ouabain-resistant) $^{86}Rb^+$ influx in density-fractionated SS discocytes and ISCs as a function of $[K^+]_0$. $[Na^+]_0$ was 150 − $[K^+]_0$. The following apparent kinetic parameters of the ouabain-sensitive $^{86}Rb^+$ influxes were obtained by plotting that data according to the logarithmic form of Hill's equation (see Materials and Methods): for the SS discocytes, K′ = 1.02, V_{max} = 2.1; for ISCs K′ = 1 10, V_{max} = 1.6.**

RESULTS

Features of experimental design. In attempting to detect a Ca^{2+}-dependent component of the passive K^+ ($^{86}Rb^+$) influx in SS cells, three experimental maneuvers were made. The basic maneuver was to expose an aliquot of each red cell sample to the ionophore A23187 in the presence of EGTA in the medium, which was shown to extract nearly all the excess Ca content of SS cells,[15] and to compare the ^{86}Rb influx in the Ca-containing and Ca-extracted cells. Most of the experiments we report here were performed on fractions of SS cells separated on Stractan density gradients. Since the dense, ISC-rich SS fraction was previously found

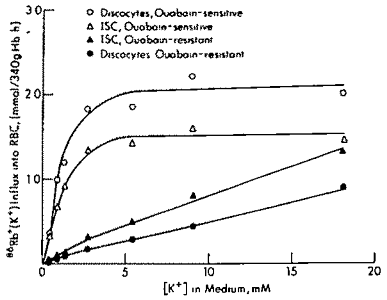

Fig 2. Effects of the removal of the endogenous cell Ca on the ouabain-resistant ^{86}Rb$^+$ influx in SS discocytes, ISCs, and unfractionated SS cells. RBCs were suspended at a 5% HCT value in media containing (in mmol/L) NaCl, 145; KCl, 5; Tris-Cl, 10, pH 7.7 (37 °C); Tris-EGTA, 1; MgCl$_2$, 0.2; glucose, 10; ouabain, 0.1; and 10 μCi/mL of ^{86}Rb. The ionophore A23187 was added from a 0.2 mmol/L solution in ethanol to give a final concentration of 1 μmol/L in the cell suspension. The percentage of lysis, determined at the end of the two-hour incubation by centrifugation and measurement of Hb concentration in the supernatant as compared with the total suspension Hb, was 3.4% and 3.3% for the unseparated cells in the absence and presence of ionophore, respectively: 1.6% and 2.8% for the discocytes; and 6.2% and 7.5% for the ISCs.

to have the highest Ca content,[5,6] the total Ca in the dense SS cell fraction from two donors was measured by atomic absorption spectroscopy before and after Ca extraction. In these samples, the total Ca contents in the dense cells were 89.0 and 71.0 μmol/340 g Hb, and after Ca extraction, 13.2 and 8.8 μmol/340 g Hb, respectively; this demonstrated that most of the excess Ca content in the ISC-rich fraction of cells was ionophore mobilizable, as observed previously in the unseparated SS cells.

Earlier analyses of the conditions for activation of the Ca^{2+}-dependent K$^+$ channel had suggested the possibility that the Ca sensitivity of the channel might be greater in ATP-depleted red cells than in fresh, substrate-fed cells.[16] Therefore, the ^{86}Rb$^+$ influx was measured in aliquots of ATP-depleted, Ca-containing and Ca-extracted red cells. Finally, as an additional condition for the ATP-depleted cells, we tested the effect of quinine, a known inhibitor of the Ca^{2+}-sensitive K$^+$ channel.[11] Since the combined addition of quinine and ouabain have been observed to produce complex effects on ion transport,[4] ouabain was omitted in cell suspensions receiving quinine additions and in the corresponding control suspension.

Table 1. Effects of Ca Extraction, ATP Depletion, and Quinine on Passive ^{86}Rb$^+$ Influx in Red Cells Incubated in High-K$^+$ Media

	^{86}Rb Influx (mmol/L RBC/hr)					
	AA Red Cells		SS Red Cells			
		ATP-Depleted		ATP-Depleted		
	Fed Ouabain	ATP-Depleted Ouabain	Fed Ouabain	Ouabain	Control	Quinine
Ca present	4 2	3 8	8 8, 5 8	8 4	7.7	8 0
Ca extracted	4 4	4 8	10 0, 7.2	8 7	8.4	7 6

RBCs were incubated in media containing (in mmol/L) KCl, 75; NaCl, 75, Tris-phosphate, 10, pH 7 5 (37 °C), Tris-EGTA, 0.1, and 10 μCi/mL of ^{86}Rb$^+$ Samples were taken at zero time (at 0 °C) and at 2, 4, 6, 10, 15, 20, 25, and 30 minutes' incubation at 37 °C and processed as described in Materials and Methods A high initial (zero time) uptake was seen with the Ca-extracted fed cells and especially with the ATP-depleted, Ca-extracted cells and was attributed to a small number of very leaky cells produced during these manipulations that equilibrated with the external ^{86}Rb$^+$ in the cold. Subsequent ^{86}Rb$^+$ uptake was linear in all samples over the 30-minute incubation period, and influx values were obtained from the slopes of lines drawn by eye through the data points When present, the concentration of ouabain was 0 1 mmol/L and of quinine, 1 mmol/L.

Passive ^{86}Rb influx in unfractionated normal and SS RBCs. Table 1 compares the passive ^{86}Rb influx in unfractionated normal and SS cells incubated in a high-K$^+$ (75 mmol/L) medium. For each condition, the ^{86}Rb$^+$ influx in the SS cells was higher than that in normal red cells (generally by about twice). But neither Ca extraction alone nor the additional procedures of ATP depletion or quinine addition revealed any indication of a Ca^{2+}-activated K$^+$ leak.

Passive ^{86}Rb$^+$ influx in density-fractionated SS RBCs Subsequent experiments were performed with density-fractionated SS cells, comparing the reticulocyte-free discocytes and the dense, ISC-rich fractions. In this group of experiments, the cells were suspended in a low-K$^+$ (5 mmol/L) medium in which any activation of the Ca^{2+}-dependent K$^+$ channel would increase the membrane potential, resulting in a proportionately larger influx of ^{86}Rb$^+$ by the countertransport mechanism.[17]

Figure 2 shows an experiment comparing the ^{86}Rb$^+$ influx in suspensions of SS discocytes, ISCs, and unfractionated SS cells. The addition of ionophore A23187 (with excess EGTA in the medium) to a parallel aliquot of each suspension caused a sharp transient increase in the ^{86}Rb$^+$ influx that was barely detectable in the discocytes and maximal in the ISCs When this response to ionophore exposure was described earlier with unfractionated SS cells,[4] it was found to be inhibited by quinine; it was therefore interpreted as an ionophore-mediated increase in the apparent affinity of the K channel for endogenous Ca, either by a direct action of the ionophore on the Ca affinity of the activation site or by its facilitating the access of Ca to the site. The new detail seen here is that the magnitude of the ionophore effect is highest in ISCs, which are also known to contain the highest Ca content.[5,6] Following this transient initial effect, however, Ca extraction had only a minimal inhibitory effect on the two-hour ^{86}Rb influx When similar experiments were performed comparing discocytes and ISCs, experiments in which the endogenous Ca was either left in the cells or was extracted with ionophore and EGTA, washing away the ionophore before the incubation, Ca extraction showed no effect on the ^{86}Rb$^+$ influx (Table 2).

Additional results in Table 2 show the passive ^{86}Rb$^+$ influx, with SS discocytes and ISCs suspended in low-K$^+$ media, under each of the conditions used to try to reveal a possible Ca^{2+}-dependent K$^+$ flux. Although some of the maneuvers were associated with small changes in the ^{86}Rb$^+$ influx, the changes bore no consistent relation to Ca extraction procedures. It is particularly noteworthy that in the dense, ISC-rich SS cell fraction, known to have the highest Ca content, neither Ca extraction nor quinine addition produced a significant decrease in ^{86}Rb$^+$ influx, and the

Table 2. Effects of Ca Extraction, ATP Depletion, and Quinine on Passive $^{86}Rb^+$ Influx in Density-Fractionated SS Red Cells Incubated in Low-K^+ Media

	^{86}Rb Influx (mmol/340 g Hb/hr)							
	SS Discocytes				Dense SS Cells (ISCs)			
	Fed	ATP-Depleted			Fed	ATP-Depleted		
	Ouabain	Ouabain	Control	Quinine	Ouabain	Ouabain	Control	Quinine
Ca present	0.42, 0.33*	0.61	0.64	0.62	0.40, 0.43*	0.32	0.40	0.62
Ca extracted	0.40, 0.28*	0.49	0.48	0.70	0.40, 0.40*	0.40	0.36	0.58

*Values were derived from the experiment illustrated in Fig 2 in which there were minor differences in the composition of the suspension media (see legend of Fig 2) and the ionophore was added to parallel suspensions after the start of the incubation to extract the cell Ca

RBCs were incubated in media containing (in mmol/L) KCl, 5, NaCl, 140, Tris-phosphate, 10, pH 7.5 (37 °C); Tris-EGTA, 0.1; and 10 μCi/mL of $^{86}Rb^+$. $^{86}Rb^+$ influx was determined as described in the legend of Table 1.

influx was not increased in the Ca-containing cells by ATP depletion.

Active and passive $^{86}Rb^+$ influx as a function of $[K^+]_0$. Measurement of the ouabain-sensitive and ouabain-resistant $^{86}Rb^+$ influx in SS discocytes and ISCs as a function of $[K^+]_0$, illustrated in Fig 1, shows that the passive K^+ ($^{86}Rb^+$) influx of the discocytes fell within a normal range,[18] whereas the ISC fraction showed an increased passive influx of $^{86}Rb^+$ that was nonsaturable over this range of $[K^+]_0$. The saturable component of the ouabain-resistant $^{86}Rb^+$ influxes, plotted separately after subtraction of the nonsaturable linear components[13] (not illustrated), was not significantly different for the discocytes and ISCs. In the experiment in Fig 1 and another similar experiment, Na pump activity, as estimated by ouabain-sensitive $^{86}Rb^+$ influx (expressed in relation to $[K^+]_0$), appeared normal in the SS discocytes ($K' = 1.02$ and 0.98; $V_{max} = 2.1$ and 2.2 mmol/340 g Hb/hr) but was inhibited in the ISC fraction, which showed normal K' values of 1.10 and 0.94 but reduced V_{max} values of 1.6 and 1.4 mmol/340 g Hb/hr

Effect of Ca extraction on active $^{86}Rb^+$ influx. In another experiment (not illustrated), ouabain-sensitive $^{86}Rb^+$ influx was measured in discocyte and ISC fractions, with an aliquot of each fraction having first been incubated with ionophore A23187 and EGTA to extract the endogenous Ca. With $[K^+]_0$ at 10 mmol/L to fully saturate the pump with K^+, the $^{86}Rb^+$ influx was 3.4 mmol/340 g Hb/hr for the discocytes and 1.0 mmol/340 g Hb/hr for the ISC fraction. Ca extraction produced no change in either value, indicating that the marked Na pump inhibition found with the ISC fraction was not corrected by removal of their excess Ca content.

DISCUSSION

The findings described here demonstrate that neither the discocyte SS cells nor the dense ISC-rich fraction, whose total Ca content generally exceeds 100 μmol/L RBCs,[5,6] show a sustained Ca^{2+}-dependent component of the passive K^+ ($^{86}Rb^+$) flux whether the cells are metabolically replete or ATP depleted.

The various predicted consequences of the established high Ca content of SS red cells, including dehydration resulting from Ca^{2+} activation of the K^+ channel,[2,3] have presumed that much if not all the excess Ca found in these cells is freely distributed in their cytoplasm If this were so, then since SS cells have been found to show cytoplasmic Ca^{2+} buffering in the normal range with a 20% to 30% ionized fraction,[15] a mean total Ca content of 40 to 60 μmol/L RBCs[15,19,20] would indicate a cytoplasmic-free ionized Ca^{2+} content of 8 to 18 μmol/L, and in the ISCs, whose total Ca content may be 100 to 200 μmol/L RBCs or more,[5,6] a Ca^{2+} of more than 20 μmol/L. Measurements of the $^{86}Rb^+$ influx in ATP-depleted normal red cells show maximal activation of the Ca^{2+}-sensitive channel above 4 μmol/L $[Ca^{2+}]_i$, with half-maximal activation around 2 μmol/L $[Ca^{2+}]_i$.[21] Levels of 1 μmol/L $[Ca^{2+}]_i$ or less in the SS cells should have produced a substantial and easily measurable increase in the $^{86}Rb^+$ influx in the SS cells. Failure to detect any Ca^{2+}-sensitive $^{86}Rb^+$ influx, even in the ISC fraction, would appear to indicate either a refractory state of the K^+ channel, which contradicts available evidence,[3,4] or compartmentalization of the Ca in SS cells in a manner that prevents its access to the inner surface of the cell membrane. Our earlier findings of the failure of SS cells to extrude much of the Ca accumulated during sickling or by ionophore loading, despite minimal abnormalities in the membrane Ca^{2+}-Mg^{2+}ATPase together with very low exchange rates of the endogenous Ca with external ^{45}Ca[15] and our initial observations of no quinine-sensitive K^+ leak in unfractionated SS cells,[4] led us to postulate that the excess Ca in these cells is compartmentalized in endocytic inside-out vesicles (EIOVs).[22] We have recently provided direct evidence to substantiate this hypothesis,[23] showing that SS cells contain vesicles that are capable of ATP-dependent Ca accumulation against an outward gradient and that the vesicles contain very high concentrations of Ca, probably in the form of precipitates with phosphates (which must be easily dissolved and extracted during brief exposure to the ionophore A23187 and EGTA).

The present findings are consistent with the compartmentalization of SS cell Ca in EIOVs. Immediately following exposure of the cells to ionophore while the Ca is being mobilized out of the vesicles and through the cytoplasm, there is transient activation of the Ca^{2+}-sensitive K^+ channel, as seen in Fig 2.

Partial inhibition of the Na pump in ISCs, as first observed by Clark et al,[7] is shown here to be characterized by normal sensitivity to $[K^+]_0$ but a low V_{max}, which is particularly abnormal in view of the elevated Na^+ concentrations in ISCs.[24,25] Clark et al found normal ATP concentrations in the

ISCs[24] as well as normal Na^+-K^+ ATPase activities in their membranes,[7] and it seemed possible that the Na pump inhibition observed in the intact ISCs might result from their high Ca content. We have shown here that the reduction in the V_{max} of the $^{86}Rb^+$ influx of the ISCs persists after extraction of their excess Ca with ionophore and EGTA. Brown and Lew[8] have found that a persistence of Na pump inhibition in red cells exposed to ionophore and Ca^{2+} is associated with an irreversible reduction in the ATP content of those cells. In view of the normal ATP concentrations found in ISCs, the persistence of Na pump inhibition after Ca extraction suggests that the increased cell Ca is not responsible for the inhibition. This interpretation is also consistent with compartmentalization of the SS Ca. If Ca had been involved in Na pump inhibition, its effects must have been irreversible and not mediated by ATP depletion.

Although the sickle cells showed no evidence of an ongoing Ca^{2+}-sensitive K^+ leak, the ouabain-resistant component of the $^{86}Rb^+$ influx, although of normal magnitude in the SS discocytes, was clearly increased in the dense, ISC-rich fraction of SS RBCs. This increase was shown to be limited to the unsaturable electrodiffusional component of the passive $^{86}Rb^+$ influx since it increased linearly with $[K^+]_0$ over the range observed. With the high outward K^+ gradient in RBCs, this increased Ca^{2+}-independent passive K^+ leak could be substantial and, if uncompensated, could contribute to the cell dehydration observed with ISCs

This finding of an increase only in the nonsaturable component of the $^{86}Rb^+$ influx of ISCs may help explain the apparent discrepancy in the results of Clark et al,[7] who described a normal passive $^{86}Rb^+$ influx in the ISCs despite an increase in the passive K^+ efflux. This increase in the K^+ efflux was similarly high in the middle and bottom SS cell density fractions when expressed as a function of the mean cell $[K^+]$ (as a rate constant). Their passive $^{86}Rb^+$ influx measurements were made at $[K^+]_0$ concentrations of 5 mmol/L, conditions under which we found little or no differences between discocytes, ISCs, and normal reported values. Our findings of an increased nonsaturable passive $^{86}Rb^+$ influx in the ISCs, which was greater than that in the discocytes, is also compatible with the observation of Clark et al of similar rate constants of a passive K^+ efflux for middle and bottom SS cell fractions since a greater leakiness in the dense cells would be counterbalanced by the lower cell $[K^+]$ in that fraction.

The absence of evidence of Ca^{2+}-sensitive K^+ channel activity in oxygenated SS cells does not exclude the possibility that transient small increases in cytoplasmic-free Ca^{2+}, which might occur during periods of increased Ca permeability associated with deoxygenation-induced Hb S polymerization and RBC sickling, could briefly activate K^+ channels in some cells, thereby contributing to their dehydration. The findings of Ohnishi[26] of an apparent Ca^{2+} dependence of the formation of dense cells during cycles of deoxygenation and oxygenation of SS cells in vitro provides some indirect evidence for such a mechanism.

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Hemoglobin Switching During Murine Embryonic Development: Evidence for Two Populations of Embryonic Erythropoietic Progenitor Cells

By Peter M.C. Wong, Siu-Wah Chung, Susan M. Reicheld, and David H.K. Chui

Explants of normal mouse embryonic tissues and disaggregated embryonic single cells were cultured in vitro to study the erythropoietic progenitor cells present during embryonic development. The results indicate that there are two populations of erythropoietic progenitor cells committed to different hemoglobin synthetic programs. These progenitor cells are present at an early gestational stage prior to the formation of the fetal hepatic primordium. One population of progenitors can be stimulated by erythropoietin alone to form usually small erythroid colonies after culture for six days in vitro. These erythroblasts primarily synthesize embryonic hemoglobins, but produce some adult hemoglobins as well. The other population of progenitors requires stimulation by both erythropoietin and adult spleen cell–conditioned medium, and usually forms large erythroid colonies after culture for six days in vitro. These erythroblasts produce only adult hemoglobins.

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ERYTHROPOIESIS during normal murine embryogenesis provides an excellent model system to study the developmental cell biology of hemoglobin switching. The circulating primitive erythrocytes, derived from yolk sac blood islands, proliferate and mature in a relatively synchronized manner. These primitive erythrocytes synthesize embryonic hemoglobins as well as some adult hemoglobins.[1-7] On day 9 of gestation, there are hematopoietic progenitor cells in the yolk sac and the embryonic circulation that, in culture, give rise to erythroblasts synthesizing adult hemoglobins.[8,9] From day 11 on, the fetal hepatic erythroblasts as well as the erythropoietic progenitor cells in the fetal livers are committed to adult hemoglobin synthesis.[10] In this study we have cultured embryonic tissues and disaggregated embryonic single cells on day 8 of gestation to document clearly the presence of different populations of erythropoietic progenitor cells during embryonic development.

The results indicate that on day 8 of gestation prior to the formation of the fetal hepatic primordium, there are two populations of erythropoietic progenitor cells. The first detectable population of progenitor cells, when cultured in vitro with erythropoietin alone for six days, usually give rise to small erythroid colonies containing erythroblasts that synthesize primarily embryonic hemoglobins but produce some adult hemoglobins as well. Addition of adult spleen cell–conditioned medium has no demonstrable effect upon the growth of these erythroid colonies. Shortly after, another population of progenitor cells were detected that, when cultured in vitro with both erythropoietin and adult spleen cell–conditioned medium for six days, usually produce large erythroid colonies synthesizing only adult hemoglobins

MATERIALS AND METHODS

Tissues and cells. Normal adult FL/4Re inbred mice obtained from the Jackson Laboratory, Bar Harbor, Me, were mated, and the morning on which the vaginal plug was found was designated as day 0 of gestation At approximately six-hour intervals on day 8 of gestation, embryos with their intact extraembryonic membranes were carefully dissected and washed free of maternal tissues and blood cells They were placed in methylcellulose culture medium[10] and cut into small fragments (<0 5 mm) before culture. On days 9 and 10 of gestation, only the yolk sacs were dissected free and cut into similarly small fragments for culture.

For disaggregated embryonic single cells, normal Balb/c mice obtained from the Cumberland View Farms (Clinton, Tenn) were mated as was described. Around noon on day 8 of gestation, embryos with their extraembryonic membranes were dissected free, washed, and digested with collagenase (Sigma Chemical Co, St Louis) at a final concentration of 0 1% in phosphate-buffered saline without calcium and magnesium but with 20% fetal calf serum (Flow Laboratories, McLean, Va) for three hours at 37 °C with intermittent agitation. At the end of the incubation, the digestion mixture was allowed to settle for five minutes, and the supernatant containing disaggregated embryonic single cells was harvested. Each day-8 conceptus would yield approximately 1 to 5 × 10^4 cells

Erythroid cell cultures Embryonic tissue fragments were cultured in methylcellulose culture medium at 37 °C for six to eight days as described previously.[10] Erythropoietin (Connaught Laboratories, Toronto; specific activity, 2 to 4 U/mg of protein) was added to these cultures, usually at 1 to 2 U/mL. Adult spleen cell–conditioned medium was prepared from C57BL/6J mice and was added to account for 20% of the erythroid cultures.[9]

Cultures of disaggregated embryonic cells were done similarly except that the spleen cell–conditioned medium was prepared according to the method of Eaves et al[11] and was added to account for 1.25% of the culture Erythropoietin was added to the cultures at a final concentration of 2 to 3 U/mL. A quantity of 1 to 3 × 10^4 cells was plated in 0.7 mL of culture medium

Hemoglobin analysis Hemolysates of the erythroblasts harvested from the cultures were analyzed by thin-layer isoelectric focusing and stained with benzidine dihydrochloride (Sigma) and scanned by a Beckman densitometer (Beckman Instruments, Inc, Burlington, Vt).[10]

For immunocytochemical studies, individual in vitro erythroid colonies were carefully picked by means of a micropipette. They were deposited on glass slides, immediately air dried, and fixed in acetone/methanol They were subsequently stained with rabbit

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anti-HbA antibodies conjugated with fluorescein and rabbit anti-HbE antibodies conjugated with rhodamine.[10] After washing, the slides were examined in a Zeiss microscope equipped for reflected light–fluorescence microscopy.

RESULTS

Cultures of embryonic tissue fragments. Early on day 8 of gestation, hemoglobinized cells were not observed in the embryonic tissues in situ. Cultures of small fragments of these early day 8 embryonic tissues and extraembryonic membranes in vitro for six to eight days with added erythropoietin produced clusters of well-hemoglobinized erythroblasts, each consisting of approximately 100 to 200 erythroid cells. All three embryonic hemoglobins (E1, x_2y_2; E11, α_2y_2; and E111, α_2z_2) were present in these cultures, but adult hemoglobins ($\alpha_2\beta_2^{\mathrm{dmaj}}$ and $\alpha_2\beta_2^{\mathrm{dmin}}$) were not detected by the isoelectric-focusing technique (Fig 1). The further addition of adult spleen cell–conditioned medium to these cultures did not alter the erythroid growths or the types of hemoglobins produced as determined by the isoelectric-focusing technique (Fig 1).

Cultures of day 8½ embryonic tissues and extraembryonic membranes with added erythropoietin produced more prominent erythroid growth. The hemoglobins produced were primarily embryonic hemoglobins, but some adult hemoglobins were also detected (Figs 1 and 2). Immunocytologic study revealed that virtually all the erythroblasts in the cultures contained both embryonic and adult hemoglobins (Figs 3A and 3B). When both erythropoietin and adult spleen cell conditioned–medium were added to the cultures of day 8½ embryonic tissues and extraembryonic membranes, there were some macroscopic erythroid bursts reminiscent of the bursts seen in cultures of days 9 to 12 embryonic peripheral blood cells[9] in addition to the well-hemoglobinized erythroid cell clusters. Both embryonic and adult hemoglobins were produced in these cultures, and the proportion of adult hemoglobins synthesized was much augmented compared to the cultures with only erythropoietin added (Figs 1 and 2). Immunocytologic study revealed that, in addition to erythroblasts containing both embryonic and adult hemoglobins, there were also many erythroblasts containing only detectable adult hemoglobins (Figs 3C and 3D).

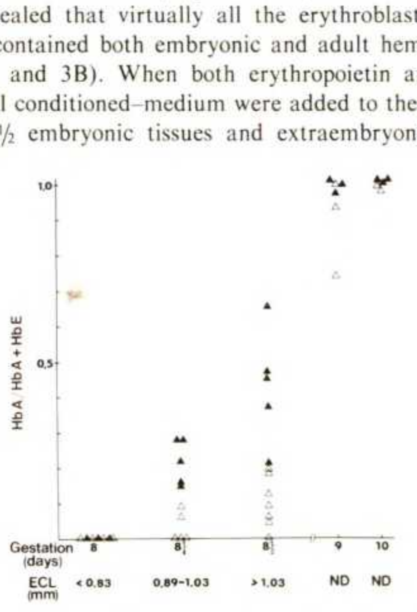

Fig 1. Proportion of HbA synthesized in cultures of fragments of FL/4Re day-8 embryonic tissues and extraembryonic membranes as well as day-9 and day-10 yolk sacs. The methylcellulose cultures were incubated for six to eight days. The hemolysates of the erythroblasts harvested at the end of the cultures were analyzed by thin-layer isoelectric-focusing and scanned by a Beckman densitometer. Each symbol represents an individual culture. (△) Cultures with only erythropoietin (Ep) added, usually at a final concentration of 2 U/mL of culture. (▲) Cultures with both erythropoietin and 20% adult spleen cell–conditioned medium (SCM) prepared from C57BL/6J mice added. Abbreviations: ECL, egg cylindrical length in millimeters measured under the dissecting microscope as an additional indication of the developmental stage of embryos used in these culture experiments; ND, not determined.

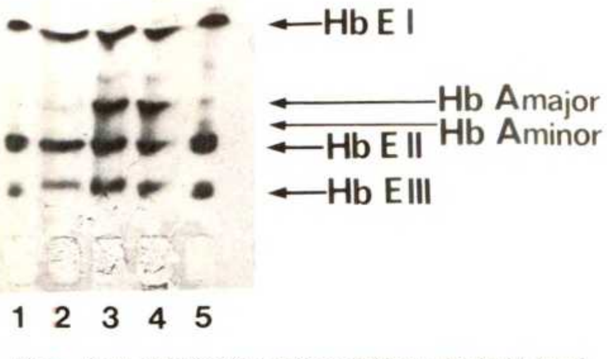

Fig 2. Isoelectric focusing of hemoglobins stained with benzidine dihydrochloride. Lanes 1 and 5, hemolysate of FL/4Re day-12 peripheral blood. Most of the erythrocytes are yolk sac blood island–derived primitive erythrocytes containing primarily embryonic hemoglobins in addition to a small amount of adult hemoglobins.[5] Lane 2, hemolysate from the culture of fragments of FL/4Re day-8½ embryonic tissues and extraembryonic membranes with only erythropoietin added. Note that there is a faint band of adult major hemoglobin in addition to that of embryonic hemoglobins. Lanes 3 and 4, hemolysates from similar cultures as in lane 2 except that both erythropoietin and adult spleen cell conditioned–medium were added. The conditioned media used were from two different perparations. Note that there are prominent bands of adult major hemoglobin in addition to that of embryonic hemoglobins.

On days 9 and 10 of gestation, the yolk sacs with many hemoglobinized primitive nucleated erythroblasts present within the vasculature were dissected free of maternal tissues and the embryo proper. These yolk sacs were further cut into small pieces and cultured. Almost all of the hemoglobins detected in these cultures were adult hemoglobins (Fig 1). Moreover, the erythroid growth was much more prominent in the cultures to which both erythropoietin and adult spleen cell–conditioned medium had been added, as has been previously reported.[9] That the cultures of days 9 or 10 yolk sac fragments produced adult hemoglobins and not embryonic hemoglobins indicates that the erythroid cell growth in the cultures after six to eight days in vitro represents the progeny of erythroid progenitor cells and not the terminal maturation of circulating primitive erythroblasts.

Cultures of disaggregated embryonic cells. Day 8½ embryonic tissues and extraembryonic membranes were digested with collagenase in the presence of 20% fetal calf serum, and single-cell suspensions were obtained. These

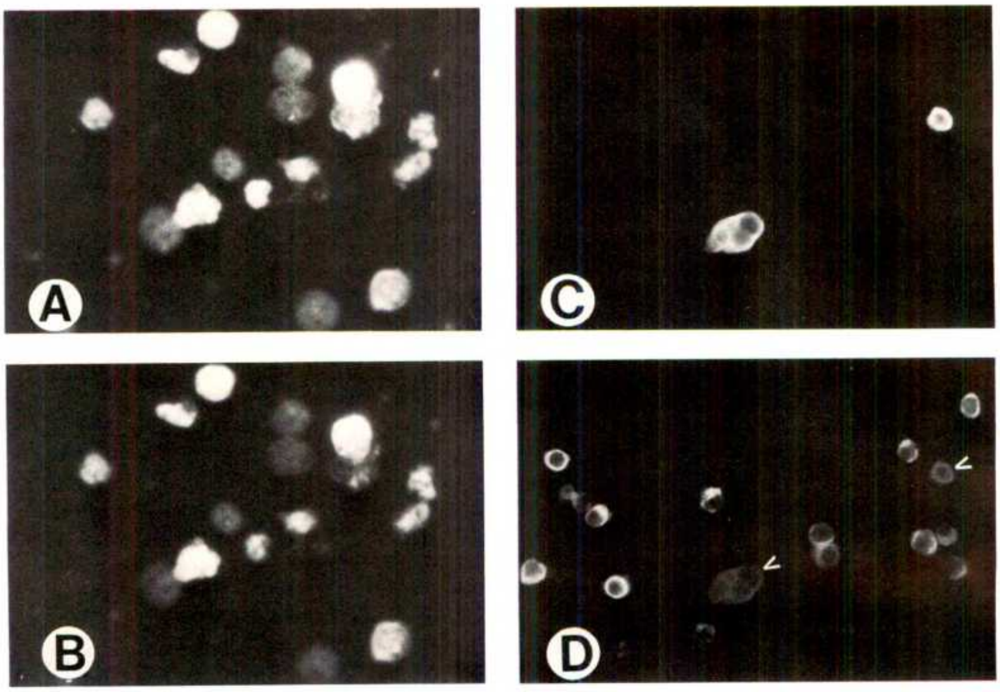

Fig 3. Immunocytologic studies of erythroblasts harvested from whole cultures of fragments of FL/4Re day-8½ embryonic tissues and extraembryonic membranes double labeled with rabbit anti-HbE and anti-HbA antibodies. Original magnification × 460; current magnification × 368. (A and B) Culture in which only erythropoietin was added. (A) Immunofluorescence of rhodamine-conjugated anti-HbE. (B) Immunofluorescence of fluorescein-conjugated anti-HbA. Note that all the cells contain both HbE and HbA. (C and D) Culture in which both erythropoietin and adult spleen cell conditioned-medium were added. (C) Immunofluorescence of rhodamine-conjugated anti-HbE. (D) Immunofluorescence of fluorescein-conjugated anti-HbA. Note that there are many cells that contain only detectable HbA in addition to cells that contain both HbE and HbA (arrow).

single cells could form erythroid colonies in vitro under the appropriate culture conditions (Table 1). Erythroid colonies were not observed in cultures to which erythropoietin and adult spleen cell conditioned-medium were not added. Erythropoietin alone stimulated the growth of many small erythroid colonies when examined after six days in culture. Adult spleen cell conditioned-medium alone produced a few small as well as large erythroid colonies. However, in cultures with both erythropoietin and adult spleen cell-conditioned medium added, there were a significant number of large erythroid colonies in addition to numerous small erythroid colonies (Table 1).

Twelve small erythroid colonies were picked from cultures in which only erythropoietin was added. These were examined by the immunocytochemical technique, and all were found to consist of erythroblasts containing both embryonic and adult hemoglobins (Table 2). On the other hand, in cultures with both erythropoietin and adult spleen cell conditioned-medium added, seven erythroid colonies, four small and three large or mixed colonies, were found to be made up

Table 1. Erythroid Colonies in Cultures of Disaggregated Embryonic Single Cells on Day 8½ of Gestation

		Colonies, No.			
Erythropoietin (2–3 U/mL)	Adult Spleen Cell Conditioned–Medium (1.25%)	Small Erythroid, <300 Hemoglobinized Erythroblasts	Large Erythroid, More Than 300 Hemoglobinized Erythroblasts	Mixed Erythroid, More Than 200 Cells Containing Hemoglobinized Erythroblasts and Other Cell Types Including Granulocytes and Macrophages	Nonerythroid, More Than 100 Cells Without Detectable Hemoglobinized Erythroblasts
No	no	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
Yes	no	263, 210, 96	0, 0, 0	0, 0, 0	14, 0, 0
No	yes	16, 16, 12	7, 4, 2	8, 7, 3	17, 11, 11
Yes	yes	305, 210, 153	24, 20, 14	16, 10, 4	26, 13, 4

Balb/c embryonic tissues and extraembryonic membranes on day 8½ of gestation were digested with collagenase in the presence of 20% fetal calf serum to obtain disaggregated embryonic single cells. The average egg cylindrical lengths of these embryos vary between 1.0 and 1.4 mm. A quantity of 2 to 4 × 10^4 cells/mL were plated in methylcellulose and cultured for six days. Colonies were enumerated under an inverted microscope without staining. Data are expressed as mean numbers of colonies per 5 × 10^4 nucleated cells plated per experiment. For each experiment, two to four methylcellulose cultures were done.

Table 2. Immunocytochemical Studies of Individual Erythroid Colonies Picked From Cultures of Disaggregated Embryonic Single Cells on Day 8 of Gestation

Experiment	Erythropoietin (2-3 U/mL)	Adult Spleen Cell-Conditioned Medium (1.25%)	Erythroid Colonies, No.		
			EA	A and EA	A
A	Yes	No	8	0	0
	Yes	Yes	2	2	4
B	Yes	No	4	0	0
	Yes	Yes	5	0	3

Disaggregated single cells of Balb/c embryonic tissues and extraembryonic membranes on day 8 of gestation were cultured in methylcellulose for six days. The average egg cylindrical lengths of the embryos used in experiments A and B were 0.9 and 1.2 mm, respectively. Individual erythroid colonies were carefully picked with a micropipette under an inverted microscope, deposited on slides, air dried, fixed, and double stained with monospecific anti-HbA antibodies conjugated with fluorescein and anti-HbE antibodies conjugated with rhodamine. Abbreviations: EA, erythroid colonies made up of erythroblasts containing both embryonic and adult hemoglobins as detected by the immunocytochemical technique; A and EA, erythroid colonies made up by many erythroblasts containing only demonstrable HbA and some other erythroblasts containing both embryonic and adult hemoglobins; A, erythroid colonies made up of erythroblasts containing only demonstrable HbA. See text for details.

of cells containing both embryonic and adult hemoglobins (Table 2 and Figs 4A and 4B). Seven other erythroid colonies, one small and six large or mixed colonies, were made up of cells containing only detectable adult hemoglobins (Table 2 and Figs 4C and 4D). There were two other large or mixed erythroid colonies that were made up of erythroblasts containing both embryonic and adult hemoglobins as well as erythroblasts containing adult hemoglobins only (Table 2).

These data are suggestive that on day 8½ of gestation there is a population of erythropoietic progenitor cells that give rise usually to small erythroid colonies after culture in vitro for six days with erythropoietin. The erythroblasts derived from these progenitor cells synthesize primarily embryonic hemoglobins, but produce some adult hemoglobins as well, similar to the yolk sac–derived primitive erythroblasts in vivo.[5] In addition, there is another population of erythropoietic progenitor cells that needs to be stimulated by both adult spleen cell–conditioned medium and erythropoietin to form usually large erythroid colonies after culture for six days in vitro. These erythroblasts produce only adult hemoglobins, similar to the fetal liver–derived erythroblasts in vivo.[10]

DISCUSSION

It has been proposed that there are two cell lineages in mammalian embryonic erythropoiesis committed to different hemoglobin synthetic programs.[12,13] The results of the present study support such a concept. Moreover, these studies show that the erythropoietic progenitor cells, capable of giving rise to progeny synthesizing primarily embryonic hemoglobins as well as progenitor cells committed to adult hemoglobin production, are detected on day 8 of gestation, prior to the appearance of the fetal hepatic primordium. A model of normal murine embryonic erythropoiesis is proposed and illustrated in Fig 5.

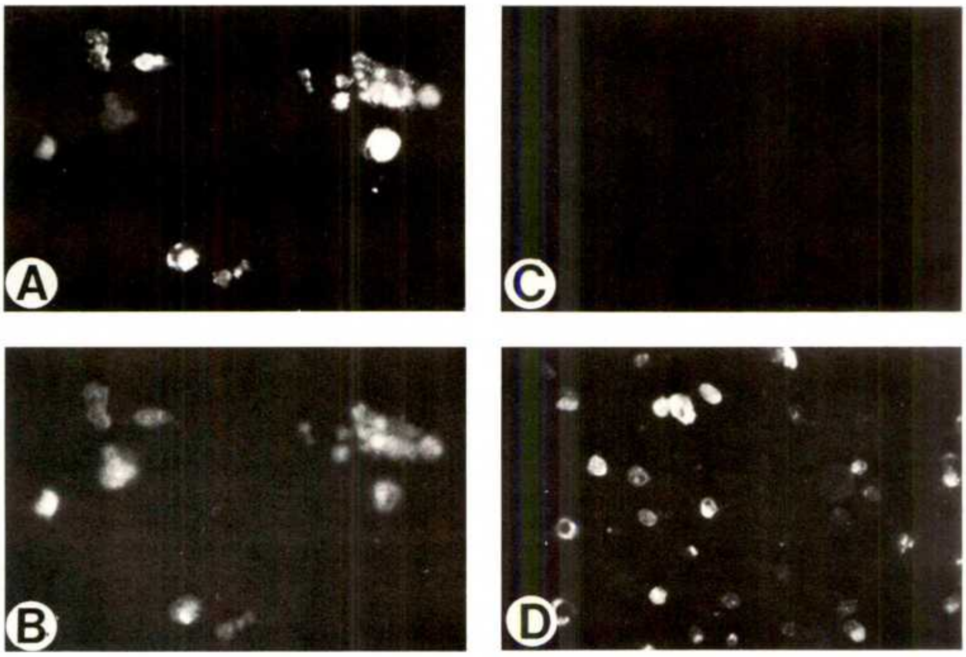

Fig 4. Immunocytologic studies of erythroblasts harvested from individual colonies in cultures of Balb/c disaggregated single-cell suspension of day-8 embryonic cells. Both erythropoietin and adult spleen cell–conditioned medium were added to these cultures. Original magnification × 380; current magnification × 304. (A and B) Small erythroid colony estimated to consist of less than 300 erythroblasts. (A) Immunofluorescence of rhodamine-conjugated anti-HbE. (B) Immunofluorescence of fluorescein-conjugated anti-HbA. Note that all the cells contain both HbE and HbA. (C and D) Large erythroid burst colony. (C) Immunofluorescence of rhodamine-conjugated anti-HbE. (D) Immunofluorescence of fluorescein-conjugated anti-HbA. Note that all the cells contain only detectable HbA.

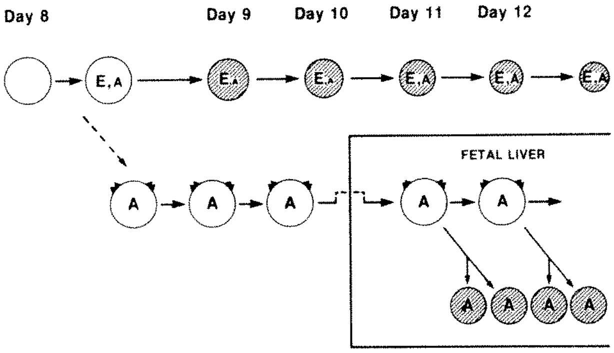

Fig 5. A model of normal murine embryonic erythropoiesis. See text. Key: ○, erythroid progenitor cells committed to the synthesis of either HbE and some HbA, or HbA; ●, Hemoglobinized erythroblasts containing either HbE and some HbA, or HbA; ▲▲, receptor for the putative mitogenic factor(s) present in the adult spleen cell–conditioned medium; ------, path not yet proven experimentally.

The first population of erythropoietic progenitor cells is found early on day 8 of gestation. More recently, we were able to detect similar erythropoietic progenitor cells by culturing disaggregated embryonic cells of day 7 of gestation. These progenitor cells usually form small erythroid colonies after culture for six days in vitro with erythropoietin alone. Adult spleen cell–conditioned medium does not have a demonstrable effect upon them. The erythroblasts in the erythroid colonies derived from these progenitor cells obtained from early day 8 embryos produced embryonic hemoglobins demonstrable by isoelectric focusing (Fig 1). It is conceivable that a very minute amount of adult hemoglobins are also present in these erythroblasts but not detectable by the comparatively less-sensitive isoelectric-focusing technique. By mid to late day 8 of gestation, these progenitor cells give rise to erythroid colonies that can be shown clearly to synthesize mostly embryonic hemoglobins but some adult hemoglobins as well, as shown by the isoelectric-focusing technique (Figs 1 and 2) and immunocytochemistry (Figs 3A, 3B, 4A, and 4B). By day 9, these erythroid progenitor cells are not found either in the yolk sac or in the embryonic circulation.[8,9]

Later, on day 8 of gestation, another population of erythropoietic progenitor cells appears whose progeny are committed to adult hemoglobin synthesis (Fig 4C and 4D). These progenitor cells are responsive to stimulation by the adult spleen cell–conditioned medium and erythropoietin (Figs 1 and 2). It is likely that the factor(s) present in the adult spleen cell–conditioned medium stimulates cellular proliferation and differentiation of erythropoietic progenitor cells already committed to adult hemoglobin synthesis. This hypothesis is supported by the following observations. First, the adult spleen cell–conditioned medium does not alter the types of hemoglobins produced in cultures of early day 8 embryonic tissues (Fig 1). Second, in the cultures of late day 8 embryonic tissues with both erythropoietin and adult spleen cell–conditioned medium added, there were in addition to the small erythroid colonies large erythroid bursts that were rarely seen in the cultures with only erythropoietin added (Table 1). Last, most of these large erythroid colonies that were found in cultures to which both adult spleen cell–conditioned medium and erythropoietin were added consisted of erythroblasts containing only adult hemoglobins demonstrable by immunocytochemistry (Fig 4C and 4D). Thus, the emergence of progenitor cells committed to adult hemoglobin synthesis is accompanied also by the appearance of receptors for the putative mitogenic factor(s) present in the adult spleen cell–conditioned medium. More definitive studies are required to rule out the other possibility that the adult spleen cell–conditioned medium has a direct effect upon the hemoglobin synthetic program of murine embryonic erythropoietic progenitor cells.

Many of the biologic activities ascribed to the adult spleen cell–conditioned medium have been found to be due to interleukin 3 (IL-3) present in the conditioned medium.[14,15] It should be informative to determine whether purified IL-3 preparation can replace the adult spleen cell–conditioned medium in stimulating the proliferation and differentiation of murine embryonic erythropoietic progenitor cells committed to adult hemoglobin synthesis. We have previously shown that embryonic fluid obtained from the exocoelom of day 10 embryos could induce the erythroid colony growths in vitro from circulating embryonic hematopoietic progenitor cells, and these erythroblasts in vitro synthesize only adult hemoglobins.[9] A recent report suggests that mouse yolk sacs can produce factors capable of inducing large erythroid colonies in vitro.[16] The nature and the mechanism of action for these activities remain to be clearly defined.

In cultures of day 8 disaggregated embryonic cells with both erythropoietin and adult spleen cell–conditioned medium added, 14 out of a total of 16 individual erythroid colonies examined were found to consist of either erythroblasts containing only adult hemoglobins or erythroblasts containing both embryonic and adult hemoglobins, as shown by the immunocytochemical technique (Table 2). The specificity of the antibodies used has been previously reported.[10] The sensitivity of the immunocytochemical technique has not been quantitatively determined. However, in a recent publication, the results obtained by the immunocytochemical studies were clearly corroborated by two different experimental approaches.[10] In the same cultures, there were two large erythroid colonies, each of which was found to consist of erythroblasts containing only adult hemoglobins and erythroblasts containing both embryonic and adult hemoglobins. It is conceivable that each of these two erythroid colonies was derived from two different erythroid progenitor cells that happened to be in close proximity to each other when first plated. Alternatively, these observations may indicate the presence of some embryonic erythropoietic progenitor cells with bipotentiality with regard to their commitment to the hemoglobin synthetic programs.

Beginning on day 9 of gestation, the erythropoietic progenitor cells committed to adult hemoglobin synthesis are found in the yolk sac and in the embryonic circulation (Fig 1) as has been previously reported.[8,9] From day 11 on, the fetal hepatic erythropoietic progenitor cells as well as the fetal hepatic erythroblasts are committed to adult hemoglobin synthesis also.[1-3,10] It is therefore probable that the circulating progenitor cells are responsible for the seeding of the fetal liver on days 10 and 11 of gestation to initiate fetal hepatic erythropoiesis, although direct and definitive experimental evidence is not yet available.

The first population of murine embryonic erythropoietic progenitor cells can be clearly detected early on day 8 of gestation. They respond to the stimulation of erythropoietin in culture and give rise usually to small erythroid colonies. Their erythroblasts produce both embryonic and adult hemoglobins. By day 9 of gestation and thereafter, these progenitor cells can no longer be detected.[8,9] On the other hand, the second population of progenitor cells was detected beginning later on day 8 of gestation. Their proliferation and differentiation in vitro require stimulation by both erythropoietin and spleen cell–conditioned medium. Usually they give rise to large or mixed erythroid colonies, and their erythroblasts synthesize only adult hemoglobins. The interrelationship of these two populations of embryonic erythropoietic progenitor cells is presently unknown. They may arise independently, or the progenitor cells committed to adult hemoglobin synthesis may evolve directly from the progenitor cells committed primarily to embryonic hemoglobin synthesis but producing some adult hemoglobins as well.

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Characterization of Natural Killer Cells With Antileukemia Activity Following Allogeneic Bone Marrow Transplantation

By Thierry Hercend, Tak Takvorian, Alexandre Nowill, Ramana Tantravahi, Philippe Moingeon, Kenneth C. Anderson, Christine Murray, Claude Bohuon, Arnaud Ythier, and Jerome Ritz

To identify cells with potential antileukemia activity following bone marrow transplantation, we have monitored immunologic reconstitution in a patient with acute lymphocytic leukemia in second remission who received intensive chemotherapy and total body irradiation followed by infusion of allogeneic histocompatible marrow. Prior to transplantation, donor bone marrow cells were depleted of T lymphocytes by in vitro treatment with anti-T12 monoclonal antibody and rabbit complement. In the first 3 weeks following bone marrow transplantation, the predominant regenerating mononuclear cell population in peripheral blood exhibited a phenotype characteristic of natural killer (NK) cells. After 4 weeks, T lymphocytes became predominant, but NK cells persisted. Cultured peripheral blood lymphocytes obtained 12 weeks posttransplant were able to display significant cytotoxicity against leukemic blasts that had been cryopreserved at the time of relapse 5 months prior to bone marrow transplantation. To further characterize those cells with antileukemia activity, we used in vitro cloning techniques to identify four monoclonal populations, termed TC12, -48, -50, and -59, with strong antitumor activity. Cytogenetic analysis demonstrated that each clone was of donor origin. Phenotypic characterization showed that the four clones expressed NKH1$_A$ but did not express T3, T4, or T8 antigens. Three of the four clones expressed T11/E rosette antigen. Each clone exhibited strong cytotoxicity against genetically unrelated hematopoietic tumor cell lines such as K562, Molt-4, JM, and U937. In addition, we found that these patient clones were similar to cloned NK cells previously derived from normal individuals. Taken together, these results suggest that at least some clones with antileukemia activity following bone marrow transplantation are cells with NK-like function and phenotype. Functional analysis of these cytolytic cells in larger numbers of patients will be necessary to determine the clinical significance of this finding.

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WITHIN the last 10 years intensive chemotherapy and total body irradiation (TBI) followed by transplantation of allogeneic bone marrow has become established as an effective and potentially curative therapeutic modality for patients with various forms of leukemia.[1-5] In using this combined modality therapy, it is widely assumed that eradication of leukemia is primarily accomplished by high-dose chemotherapy and TBI and that the transplantation of allogeneic marrow simply acts in a supportive role to reconstitute marrow function that has been irreversibly damaged by the ablative treatment program. In addition, it has been postulated that immunologic reconstitution with allogeneic cells may in itself have a significant antileukemic effect. This view is supported by the recent finding that those transplant recipients in whom significant graft-*v*-host disease (GVHD) developed have a decreased risk of leukemia relapse compared to patients in whom GVHD does not develop.[6,7]

The present studies were undertaken to investigate the possible existence of cells with direct cytotoxicity for leukemic cells following bone marrow transplantation (BMT) and to determine whether this activity could be distinguished from GVHD. In so doing, we undertook phenotypic and functional characterization of peripheral blood lymphocytes (PBL) regenerating following allogeneic BMT in a single patient with T cell acute lymphoblastic leukemia (T-ALL) from whom we had been able to cryopreserve leukemic blasts at the time of relapse 5 months prior to transplantation. Using recently developed methods for establishing and expanding cloned cytotoxic cells without preselected specificity,[8,9] we identified four distinct clonal cell lines capable of mediating cytotoxicity against the patient's leukemic blasts. Further characterization of these cloned cell lines demonstrated that all exhibited the phenotype of natural killer (NK) cells as well as functional NK activity.

MATERIALS AND METHODS

Monoclonal antibodies. All monoclonal antibodies used in this study have been described previously. Briefly, anti-T3 defines all mature T lymphocytes.[10] T4 antigen is expressed on a subset of peripheral T cells having primarily inducer function.[11] T8 antigen is expressed on a subset of peripheral T cells having primarily cytotoxic/suppressor function.[12] Anti-T11 defines an antigen associated with the sheep erythrocyte receptor.[13] T12 antigen is expressed on a fraction of thymocytes and the vast majority of peripheral blood T cells.[13] Anti-NKH1$_A$ defines a pan-NK cell antigen that is expressed on all resting as well as activated NK cells and NK clones.[14] Anti-NKH2 characterizes a subset of large granular lymphocytes (LGL) in peripheral blood and is only expressed on some NK active clones.[14] Anti-B1 reacts with all normal B cells,[15] and anti-MY4 reacts with all peripheral blood monocytes.[16] Anti-T3, -T4, -T8, -T11, -T12, -B1, -MY4, and -NKH1$_A$ antibodies are available from Coulter Immunology, Hialeah, Fla.

Phenotypic analysis of cell surface antigens. Phenotypic analysis was performed by indirect immunofluorescence with fluorescein-

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conjugated goat antimouse Fab IgG (Meloy or Tago) as previously described.[8,9] Samples were analyzed on an Epics V (Coulter Electronics, Hialeah, Fla) or a FACS 440 (Becton Dickinson, Mountain View, Calif) flow cytometer. Ten thousand cells were analyzed in each sample. Negative controls used to determine background fluorescence were ascites derived from a nonreactive hybridoma. Monoclonal antibodies were always used at saturating concentrations (1:500 to 1:1,000).

Cytotoxicity assays. Cytotoxicity assays were performed according to a standard chromium release method previously described.[8] All experiments were done in triplicate using V-bottom microtiter plates. Cytotoxicity assays were performed usually for three hours at 37 °C using 5,000 target cells/well. Medium was RPMI 1640 plus 5% pooled human AB serum and 1% penicillin-streptomycin. Target cells in these assays included Molt-4 and JM, which are T cell leukemia cell lines. K562 was derived from a patient with chronic myelogenous leukemia. U937 is a histiocytic cell line. Laz 509 is an Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell line.

Preparation of lymphocyte-conditioned media. Lymphocyte-conditioned medium (LCM) was produced by stimulation of peripheral blood mononuclear cells (PBMC) at a concentration of 2×10^6 mL with 4 μg/mL phytohemagglutinin (Burroughs Wellcome, Greenville, NC), 5 μg/mL phorbol myristate acetate; (Sigma Chemical Co, St Louis), and irradiated (5,000 rad) EBV-transformed B lymphoblastoid cells (PBMC/irradiated EBV-transformed B cells ratio, 3:1). Following a first incubation for three hours at 37 °C, cells were washed four times to remove the mitogens and resuspended in RPMI 1640 supplemented with 2.5% human AB serum. After 40 hours of subsequent incubation at 37 °C, culture supernatants were harvested, passed through 0.22-μm filters and stored at −70 °C.

Generation of cloned cell lines. Methods for generation of human NK cloned cell lines have been previously described in detail.[8,9] Briefly, clones were obtained using a limiting-dilution technique at 0.5 cells/well. Cell lines were generated using the same experimental conditions, but cloning was done at 100 cells/well. Cells were cloned on a feeder layer of allogeneic irradiated PBMC (5,000 rad) plus irradiated EBV-transformed B cells (Laz 509). Initial expansion of the colonies was ensured by the addition of 10% LCM every three or four days. Culture medium was RPMI 1640 supplemented with 1% penicillin-streptomycin, 1% sodium pyruvate, and 15% human AB serum.

Cytogenetic analysis. Bone marrow cells from the patient obtained prior to transplantation were cultured for 24 hours at 37 °C. Cells were first treated with 0.1 μg/mL Colcemid (GIBCO, Grand Island, NY) for ten minutes and then with 75 mmol/L KCl for 20 minutes at 37 °C. Following hypotonic treatment, cells were centrifuged and the cell pellets broken up and fixed in 3:1 (vol/vol) methanol:acetic acid for one hour. After two changes of fixative, standard air-dried slides were prepared. Peripheral blood cultures from the donor were set up in media containing PHA, incubated for 72 hours at 37 °C, and processed as previously described. Cultured cell lines were treated with 0.01 μg/mL Colcemid for 30 minutes, treated with hypotonic KCl for ten minutes at 37 °C, and processed as previously described. All slides were stained with quinacrine mustard (25 μg/mL), rinsed in running tap water, and mounted in Tris-maliate buffer, pH 5.6. Fluorescence-banding patterns were recorded on Kodak Technicolor Pan film using a Leitz Ortholux fluorescence microscope equipped with an Orthomat camera.

CASE REPORT

This 32-year-old male was initially found to have acute lymphocytic leukemia in Dec 1980. At that time his primary complaint was chest pain and dyspnea, and chest x-ray results demonstrated a large anterior mediastinal mass. An examination of the bone marrow demonstrated a replacement of normal marrow cells with a diffuse infiltrate of lymphoblasts. Remission induction was achieved following the administration of Adriamycin, vincristine, prednisone, and L-asparaginase. He subsequently received CNS irradiation and intrathecal methotrexate and maintenance chemotherapy with intermittent cycles of prednisone, vincristine, methotrexate, and 6-mercaptopurine. Adriamycin was added to the maintenance regimen every 3 months. After 36 months of therapy, examination of the peripheral blood and bone marrow demonstrated a leukemia relapse. The peripheral WBC count was 120,000/μL, with almost all cells being lymphoblasts. The bone marrow was markedly hypercellular and was again almost totally replaced with lymphoblasts. The phenotype of bone marrow lymphoblasts as determined by indirect immunofluorescence assay and cell sorter analysis was as follows: T11+, T6−, T3 weakly +, B4−, B1−, common acute lymphoblastic leukemia antigen-negative, MY9−. Further treatment with vincristine, prednisone, Adriamycin, and L-asparaginase was able to induce a second remission.

While still in second remission, he underwent allogeneic marrow transplantation with cells from his histocompatible brother (HLA-A, -B, -C, -D identical and mixed lymphocyte culture nonreactive). Prior to infusion of marrow he received intensive therapy consisting of the following: cytosine arabinosine, 3 g/m^2 intravenously (IV) (four doses on days −5 and −4), cyclophosphamide, 60 mg/kg IV (two doses on days −5 and −4), TBI, 1,200 rad (200-rad fractions twice daily on days −3, −2, and −1). To prevent GVHD, donor marrow was treated in vitro with anti-T12 monoclonal antibody and rabbit complement to remove mature T cells.[17] The total number of cells infused was 2.2×10^9 (2.7×10^7 cells/kg). No other GVHD prophylaxis was administered. This protocol was approved by the Institutional Review Boards of the Dana-Farber Cancer Institute and Brigham and Women's Hospital. Before informed consent was obtained, the procedure and potential risks and benefits were explained to the patient and his family.

Hematopoietic reconstitution posttransplantation proceeded without complications. Nucleated RBCs were first seen in peripheral blood on day 8. Circulating granulocytes rose to levels >500/μL by day 17. The platelet count remained >20,000 without platelet transfusions after day 14. By day 19 posttransplant, the WBC count was 2,600/μL with 42% granulocytes, the hematocrit value was 37%, and the platelet count was 38,000/μL. Engraftment with allogeneic cells of donor origin was documented by cytogenetic analysis that identified chromosomal polymorphisms of donor type in metaphase preparations of marrow and stimulated peripheral blood lymphocytes posttransplantion. Acquisition of the donor-type red cell antigen phenotype was also documented posttransplant. He was discharged on day 20 after marrow transplantation. Following discharge, his clinical course was complicated by the development of right upper-quadrant abdominal pain, ascites, pedal edema, and a mildly elevated bilirubin level (total, 2.0 mg/dL). This was felt to be secondary to veno-occlusive disease of the liver and responded rapidly to treatment with oral prednisone (40 mg/d). During his early posttransplant course, mild skin erythema developed that resolved without specific treatment by day 20. Diarrhea or other manifestations of GVHD never developed. At present, he continues in remission 20 months posttransplant.

RESULTS

Phenotypic analysis of PBMC posttransplantation. At frequent intervals posttransplantation, PBMC were obtained and analyzed for reactivity with a series of monoclonal antibodies defining antigens expressed by normal T cells, B cells, NK cells, and monocytes. Results of this analysis on days 11, 18, 29, and 58 post-BMT are shown in Fig 1. In the first 3 weeks post-BMT, cells expressing NKH1$_A$ represented the major population of early reconstituting mononuclear cells. Two-color immunofluorescence assays demonstrated that the NKH1$_A$-positive cells also expressed T11/E rosette antigen but did not express T3 antigen (data not shown). Although a minor population of mature T cells expressing T3, T11, and T4 or T8 antigens were also present during this time, there were very few cells expressing T12 antigen, which is also found on normal mature T cells in peripheral blood. By 4 weeks post-BMT, the percentage of NKH1$_A$-positive cells had decreased, and the percentage of cells expressing T3, T11, and T4 or T8 antigens had increased so that T lymphocytes now represented the major population of PBMC. At this time, T cells were also found to express T12 antigen and thus displayed a more normal pattern of T cell surface markers. A high level of NK activity was maintained even after T lymphocytes became the predominant mononuclear cell in peripheral blood. When tested against K562 target cells, uncultured PBMC obtained 34 days post-BMT exhibited a 48% specific cytotoxicity at 60:1 effector/target cell (E/T) ratio, 35% at 30:1, and 22% at 15:1.

Circulating B cells identified by reactivity with anti-B1 first appeared 9 weeks post-BMT. A moderate degree of monocytosis was evident by reactivity with anti-MY4 on days 18 and 29 but did not persist after 4 weeks. During the first 3 months post-BMT, cells expressing the Ia antigen consistently represented >50% of PBMC (data not shown), suggesting that these cells were activated and proliferating in vivo. Expression of the NKH2 antigen varied between 10% and 29% of PBMC.

Generation of polyclonal lymphocytic cell lines pre- and post-allogeneic marrow transplantation. In conjunction with the phenotypic and functional characterization of lymphocyte reconstitution post-BMT, polyclonal lymphoid cell lines were established from peripheral blood obtained before and after allogeneic transplantation using in vitro stimulation and subsequent expansion with interleukin 2 (IL 2). The lymphoid cell line developed from pretransplant cells (termed TL-pre) was generated from a vial of PBMC cryopreserved at the time of leukemic relapse, 5 months prior to transplantation. Generation of this cell line was based on the assumption that a small percentage of normal T lymphocytes was still present in the peripheral blood during the acute phase of the disease. Thawed cells were stimulated with concanavalin A and cultured for 2 weeks in the presence of LCM containing IL 2. After 2 weeks, leukemic blasts were no longer detectable in the culture. Remaining viable cells were then expanded by plating 100 cells/well onto a feeder cell layer of irradiated normal PBL and EBV-transformed B cell line as described in Materials and Methods. The phenotype of this cell line is summarized in Table 1. TL-pre cells were primarily mature T lymphocytes expressing T3, T11, and T12 antigens. This cell line contained approximately equal percentages of T4+ (37%) and T8+ (32%) cells and relatively small percentages of cells expressing NKH1$_A$ (13%) and NKH2 (15%) antigens. This phenotype was clearly different from that of the T-ALL blasts, which expressed T3 antigen only weakly and did not express either T4 or T8 antigens.

Table 1. Phenotypic Analysis of Polyclonal Cell Lines Derived From Peripheral Blood Prior to and Following BMT

Cell Line	T3	T4	T8	T11	T12	NKH1$_A$	NKH2
TL-pre	86*	37	32	99	92	13	15
TL-post	66	5	73	94	33	47	35

*Percentage of cells expressing antigens defined by monoclonal antibodies.

The posttransplant cell line (termed TL-post) was established from peripheral blood obtained 83 days post-BMT and was subsequently used to develop cloned cell populations for the identification of cells with antileukemic activity. TL-post was obtained following stimulation and expansion of PBMC in LCM until sufficient numbers of cells were available for phenotypic and functional characterization. The phenotype of the TL-post cell line is summarized in Table 1. The great majority of these cells expressed T11 antigen, but in contrast to TL-pre cells, fewer cells were T3+, almost all cells were T8+, and very few were T4+. Expression of T12 antigen was less than expression of T3 antigen and was considerably weaker than in TL-pre cells. In addition, a greater percentage of the TL-post lymphocytes expressed the NK cell markers NKH1$_A$ (47%) and NKH2 (35%). More importantly, TL-post lymphocytes displayed significant activity against the patient's cryopreserved leukemic cells (23% specific cytotoxicity at 60:1 E/T ratio).

Generation of cloned cell lines displaying antileukemic activity. Cells obtained from the polyclonal TL-post cell line were cloned at 0.5 cells/well using procedures described in Materials and Methods. Clonal expansion was maintained by the addition of LCM every three days. Between 2 and 3 weeks following cloning, 74 macroscopic colonies were apparent (cloning efficiency, 30%). Cloned colonies were individually screened for cytotoxicity against uncultured freshly thawed leukemic blasts in a conventional four-hour ^{51}Cr release assay. Of the 74 colonies, six displayed cytotoxicity against the leukemic blasts. These six colonies were

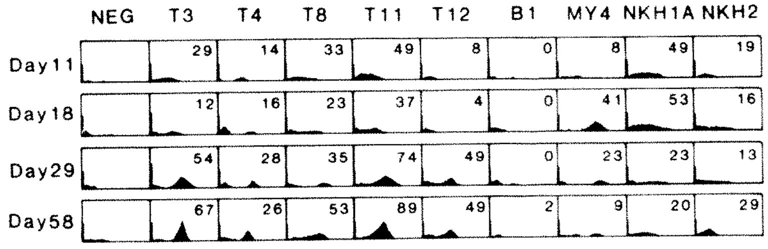

Fig 1. Phenotype of PBMC following allogeneic transplantation with anti-T12-depleted bone marrow. PBMC were analyzed on days 11, 18, 29, and 58 posttransplant. Each display represents a two-dimensional histogram plotting intensity of fluorescence on a log scale (x-axis) *v* cell number on a linear scale (y-axis) for each antibody tested. The numbers in each box represent the percentage of positive cells above the background for each reagent.

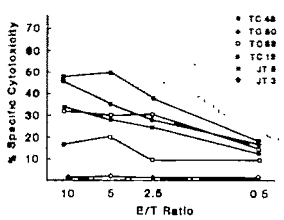

Fig 2. Cytotoxicity of clones TC12, TC48, TC50, and TC59 for leukemic T cell blasts. Effector cells (E) were incubated with target cells (T) at various ratios for three hours at 37 °C.

subcloned at 1,000 cells/well using experimental conditions identical to the initial cloning to rapidly obtain sufficient numbers of cells for further phenotypic and functional characterization. Before being discarded, all colonies that did not display cytotoxicity for the patient's leukemic cells were tested for conventional NK activity using K562 target cells. This second screening procedure identified an additional nine colonies that were strongly cytotoxic (>20% specific killing) against K562 cells but had no detectable antileukemic activity. Of the six colonies with antileukemic activity, four displayed good proliferative capacity following subcloning procedures and could be further studied in detail. These clones were termed TC12, -48, -50, and -59.

Following in vitro expansion, clones TC12, -48, -50, and -59 were retested several times against freshly thawed uncultured leukemic blasts. As shown in Fig 2, each of the four expanded clones exhibited cytotoxicity against the leukemic cells that was still evident at low E/T ratios consistent with the data obtained in the initial screening procedure. Two previously described human NK clones termed JT3 and JT9 were also used as effector cells in this representative experiment.[9] As shown, JT9 cells had a low level of cytotoxicity against the leukemic blasts, whereas JT3 cells had no activity at all. Cytocentrifuge smears of thawed target cell preparations confirmed that these target cells contained >80% leukemic blasts.

Cytotoxicity of patient clones against conventional NK target cell lines. The cytotoxic activity of the four selected clones was tested against a series of in vitro established hematopoietic cell lines that are commonly used as target cells in conventional NK assays. As shown in Table 2, the four clones were cytotoxic against K562, Molt-4, JM, and U937 cells. In contrast, there was no activity against the EBV-transformed B cell line Laz 509. These results are similar to those obtained with a series of previously described NK clones that display broad reactivity against hematopoietic cell lines but have relatively little activity against EBV-transformed B cells.[9,14]

The TC clones were also tested against normal activated T lymphocytes derived from peripheral blood cells of the patient. For this purpose, lymphocytes from the TL-pre cell line were used as target cells. It was found that TC12, -48, -50, and -59 had no cytotoxic activity against normal activated T lymphocytes autologous to the T cell leukemic blasts (Table 2).

Phenotypic analysis of clones TC12, -48, -50, and -59. Phenotypic analysis of antileukemic clones was performed using indirect immunofluorescence assays and subsequent analysis by flow cytometry. The clones were tested with a series of monoclonal antibodies that have been previously used to characterize NK cloned cell lines derived from normal donors and are included for comparison in Table 3. TC48 cells were found to express $NKH1_A$ antigen but did not react with anti-T3, -T4, -T8 or -T11. This pattern of reactivity appeared to be similar to that of a previously described clone termed JT1.[8,14] However, as opposed to JT1 cells, TC48 cells did not express NKH2 antigen. It was also found that TC48 cells did not react with anti-$T11_2$ and anti-$T11_3$ antibodies, which define other epitopes of the E rosette receptor molecule[18] (data not shown). Clone TC59 was found to express T11 as well as $NKH1_A$ and NKH2 antigens, a phenotype similar to that of the cloned NK cell line JT3.[14] Clones TC12 and TC50 displayed an identical phenotype using this series of antibodies. These two latter clones were found to express T11 and $NKH1_A$ but did not react with anti-NKH2 and were thus similar to the NK clone JT_B18.[14] None of the clones expressed the T cell antigens T3, T4, or T8.

Cytogenetic characterization of clones TC12, -48, -50, and -59. Quinacrine bright polymorphisms were compared between metaphases obtained from the patient's bone marrow cultures and PHA-stimulated leukocyte cultures from the donor. One informative polymorphism was observed. Although both copies of chromosome 21 from the patient lacked any polymorphism (Fig 3A), one of the copies of chromosome 21 in the donor had quinacrine bright satellites (Fig 3B). Twenty-seven Q-banded metaphases from the four clones were analyzed. All of them had a chromosome 21 with the bright satellites (Fig 3C), indicating that the clones are derived from donor cells.

DISCUSSION

In the present study, peripheral blood cells from a single individual were analyzed at various times during immunologic reconstitution following allogeneic BMT with T cell–depleted marrow. The primary goal of this characterization was to identify those cells that might be capable of exerting a

Table 2. Cytotoxicity of Antileukemic Clones Against Conventional NK Target Cell Lines and Normal Activated T Cells

	E/T ratio											
	K562		Molt-4		JM		U937		Laz 509		TL-pre	
Clone	6:1	2:1	6:1	2:1	6:1	2:1	6:1	2:1	6:1	2:1	10:1	5:1
TC 12	32*	27	69	44	84	75	42	50	3	2	1	0
TC 48	24	13	34	22	51	38	39	60	2	1	2	1
TC 50	37	31	59	59	72	50	35	36	2	2	0	0
TC 59	35	23	47	31	45	32	18	9	2	2	2	2

*Percent specific cytotoxicity, mean of triplicate assays, SD ≤5%.

Table 3. Antigenic Phenotype of Cloned Cell Lines Mediating Antileukemic Activity: Comparison With NK Clones Derived From Normal Donors

Clone	T3*	T4	T8	T11	$NKH1_A$	NKH2
JT1†	−	−	−	−	+	+
TC48	−	−	−	−	+	−
JT3†	−	−	−	+	+	+
TC59	−	−	−	+	+	+
JT_B18†	−	−	−	+	+	−
TC12	−	−	−	+	+	−
TC50	−	−	−	+	+	−

Key: −, <5% of cells reactive; +, >90% of cells reactive.

*Expression of antigens determined by indirect immunofluorescence reactivity with specific monoclonal antibodies.

†Derived from normal donor.

direct cytotoxic effect against the patient's leukemic blasts. In this regard, we found that the major population of PBMC reconstituting during the first 3 weeks post-BMT had an NK cell phenotype and exhibited high levels of NK activity. Only after 1 month did large numbers of T cells develop. At 12 weeks post-BMT, the peripheral blood still contained 24% $NKH1_A$-positive cells, and a polyclonal cell line established at this time was able to mediate a relatively low but significant level of cytotoxic activity against freshly thawed leukemic cells that had been cryopreserved at the time of relapse. To identify the cells responsible for this antileukemic activity, cultured cells were cloned at 0.5 cells/well using techniques that had previously been developed to establish clonal populations of NK-active cells from normal individuals. Using these techniques, four different clonal cell lines with antileukemic activity could be identified and propa-

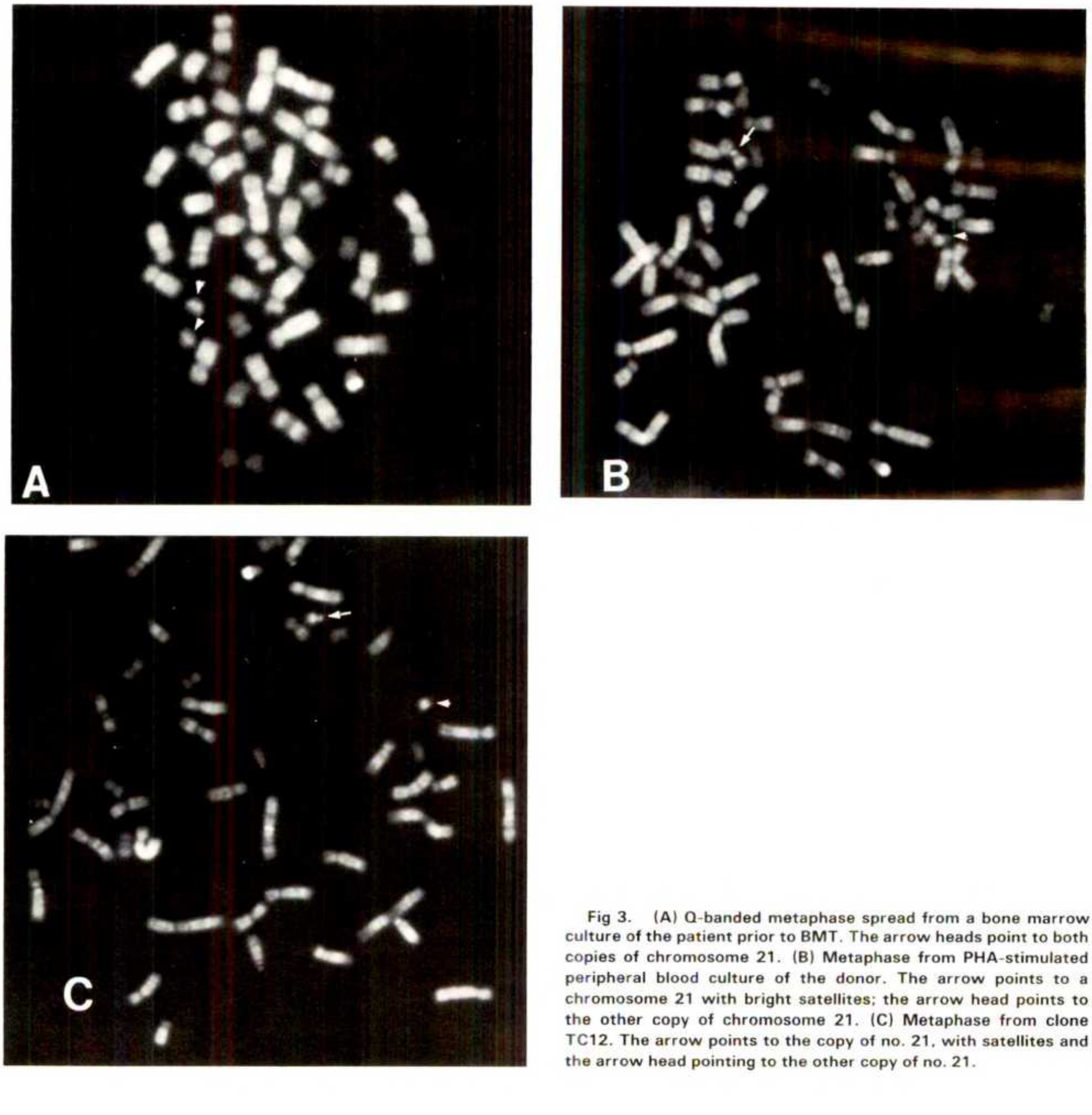

Fig 3. (A) Q-banded metaphase spread from a bone marrow culture of the patient prior to BMT. The arrow heads point to both copies of chromosome 21. (B) Metaphase from PHA-stimulated peripheral blood culture of the donor. The arrow points to a chromosome 21 with bright satellites; the arrow head points to the other copy of chromosome 21. (C) Metaphase from clone TC12. The arrow points to the copy of no. 21, with satellites and the arrow head pointing to the other copy of no. 21.

gated in sufficient numbers to allow a detailed phenotypic and functional characterization. Cytogenetic analysis confirmed that each clone was derived from the HLA-compatible allogeneic donor. Immunofluorescence analysis of cell surface markers demonstrated that each clone had a characteristic phenotype representative of NK cells and that these phenotypes were similar to those of NK clones previously derived from normal individuals. Finally, each clone was cytotoxic for standard NK target cells as well as for leukemic blasts, but these clones did not exhibit cytotoxicity for normal activated T cells derived from the patient prior to BMT.

Taken together, the clonal analysis of cytotoxic cells in our patient suggests that activated NK cells derived from the donor may be capable of exerting a direct antileukemic effect following allogeneic marrow transplantation. This conclusion is consistent with the results of experiments in animal models that have suggested that NK cells play a role in normal immune resistance to radiation-induced leukemias as well as other hematopoietic and nonhematopoietic tumors.[19] In the context of marrow transplantation, however, NK cells have been implicated in a number of sometimes conflicting responses. For example, several studies have demonstrated that NK cells in the host can mediate immunologic resistance to an allogeneic marrow graft.[19-21] It has also been suggested that NK cells in the allogeneic graft play a role in producing GVHD,[22-24] whereas other studies have found no correlation between NK activity post-BMT and either leukemia relapse or GVHD.[25] The implication of NK cells in such diverse activities may reflect the extensive phenotypic and functional heterogeneity of these cells. In previous studies, we and others have established cloned cell lines that maintain NK activity from normal peripheral blood and have used these cultured cells to characterize NK activity at the single-cell level.[9,19,26,27] The diversity of phenotypes and functional specificity demonstrated with these cloned NK cell lines appeared to reflect the heterogeneity of uncultured cells capable of mediating NK function, and several of these cloned cell lines were used for comparative purposes in the present analysis. Of interest, several normal NK clones were either not able to kill our patient's cryopreserved leukemic cells or had very low levels of activity even though their phenotype and cytotoxicity for other NK targets was similar to that seen with clones TC12, -48, -50, and -59. Moreover, it should also be pointed out that the ability to kill leukemic targets was not shared by all NK-active clones established post-BMT. In fact, only six of 15 clones having a high degree of cytotoxicity for K562 were able to kill leukemic targets, again demonstrating the extensive heterogeneity of cells capable of mediating NK-like functions.

In part, our clonal analysis of cytotoxic cells post-BMT was facilitated by the method we used to prevent GVHD. More specifically, this patient received allogeneic marrow that was depleted of mature T cells by in vitro treatment with anti-T12 antibody and rabbit complement.[17] He did not receive any other GVHD prophylaxis. T12 antigen is normally expressed on functionally mature T lymphocytes in peripheral blood and thymus but is not expressed on less-differentiated T cells within the thymus and, more importantly, is not expressed to any large extent on normal NK cells. The early proliferation of NK cells and delayed proliferation of mature T cells in our patient therefore likely reflects the in vitro treatment with anti-T12, which is more effective in depleting T cells than NK cells. NK cells normally reconstitute in the early posttransplant period and have been associated with acute GVHD, but NK cell proliferation would occur in the context of rapid T cell proliferation if T cells were not depleted from the donor marrow prior to transplantation.[28] The finding that NK cells can reconstitute in large numbers without the development of clinically significant GVHD suggests that these cells are not responsible for GVHD. The additional finding that cloned NK cells from the donor are capable of cytotoxicity against the recipient's leukemic cells further suggests that it may be possible to distinguish graft-*v*-leukemia from GVHD following human marrow transplantation as has previously been demonstrated in murine experiments.[29]

Although the present studies suggest that donor NK cells may play a role in preventing leukemia relapse after marrow transplantation, it should be emphasized that our studies were carried out in a single patient with T-ALL. We have not yet analyzed NK cells in other allogeneic transplant recipients with different types of leukemia or following autologous or syngeneic transplantation. The clonal analysis of cytotoxic effector cells is a laborious method that cannot easily be applied to large numbers of patients, but similar studies will have to be carried out in additional patients before the clinical significance of NK cells post-BMT is fully understood.

ACKNOWLEDGMENT

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Acid Phosphatase Activity in Mononuclear Phagocytes and the U937 Cell Line: Monocyte-Derived Macrophages Express Tartrate-Resistant Acid Phosphatase

By Rose G. Snipes, K.-W. Lam, Richard C. Dodd, T. Kenney Gray, and Myron S. Cohen

Tartrate-resistant acid phosphatase (TRAcP) is used as a marker for osteoclasts, which are believed to be derived from phagocytic cells or phagocyte stem cell precursors. To further investigate the relationship between monocytic phagocytes and osteoclasts, acid phosphatase (AcP) activity was measured by three different techniques in human peripheral blood monocytes, monocyte-derived macrophages, and the U937 cell line. We found that cytochemistry and gel electrophoresis led to similar results, but that the colorimetric assay was inconsistent. Normal human peripheral monocytes expressed both tartrate-sensitive and -resistant AcP. In culture these cells formed polykaryons and expressed TRAcP activity that was further identified as an isoenzyme associated with bone tissue. In contrast, the U937 cells did not express TRAcP activity as measured by gel electrophoresis. Both U937 cells and monocytes possess material that interferes with interpretation of the colorimetric assay of AcP. The presence of TRAcP in monocyte-derived macrophages further supports the relationship between phagocytic cells and bone osteoclasts.

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ACID phosphatases (AcP)[1] are enzymes found in all body tissues including human leukocytes.[1-3] These enzymes hydrolyze monophosphate esters in an acid environment. AcP activity can be demonstrated in individual cells by histochemical techniques[4,5] or in extracted tissue by either colorimetric reactions[1] or acrylamide gel electrophoresis.[6,7] These enzymes are divided into two major groups based on their sensitivity to tartrate inhibition. Most of the AcP in normal serum is tartrate resistant.[8] Tartrate-resistant acid phosphatase (TRAcP) is associated with bone tissue,[9-11] the neoplastic cells of leukemic reticuloendotheleosis (hairy-cell leukemia[12-14]), and spleen cells from patients with Gaucher's disease.[14-16] TRAcP activity has been identified by cytochemical stain in multinucleated giant cells of bone (osteoclasts), and this enzyme is regularly used as a marker for osteoclasts.[10,17]

The mechanisms by which monocytic phagocytes (or their precursors) differentiate into osteoclasts remain unknown. Furthermore, because of the difficulty in obtaining sufficient monocytes for study as well as their maturity after release from the bone marrow, we and others have used a variety of cell lines for studies of monocyte differentiation.[7,18-20] U937, a cell derived from a histiocytic lymphoma,[21] will differentiate into a macrophagelike cell[7,18,20] and form multinucleated giant cells (polykaryons) under appropriate conditions of incubation. Because of the significance of TRAcP activity to the operational definition of the osteoclast,[10,17] we conducted detailed studies of the AcP chemistry of human peripheral blood monocytes, monocyte-derived macrophages, and the U937 cell line. Our results demonstrate major differences between human monocytes and the U937 cell line. Furthermore, we have identified the TRAcP isoenzyme associated with bone tissue and osteoclasts in monocyte-derived macrophages.

MATERIALS AND METHODS

Reagents

1,25-dihydroxyvitamin D_3 ($1{,}25(OH)_2D_3$) was obtained as a gift from Milan Uskokovic, PhD, Hoffmann-LaRoche, Nutley, NJ. The $1{,}25(OH)_2D_3$ was dissolved in 100% ethanol and added to cell cultures to give a final ethanol concentration of 0.1%. In all experiments, appropriate controls were performed to exclude an ethanol effect.

Isolation and Preparation of Cells

Human monocytes. Venous blood was obtained from normal volunteers consistent with the guidelines of the Human Rights Committee of the University of North Carolina at Chapel Hill. Human monocytes were prepared as previously described[22] by placing heparinized whole blood on a Plasmagel (Cellular Products, Buffalo) sedimentation gradient. The white cell layer was removed, centrifuged, and the pellet diluted in phosphate-buffered saline and placed on a Ficoll-Hypaque gradient (specific gravity 1.077, Sigma Chemical Co, St Louis, and Winthrop Laboratories, New York). Cells were centrifuged at 150 g for 35 minutes. The mononuclear layer was removed, centrifuged at 200 g for 10 minutes, and subsequently washed three times in alpha-modified minimal essential medium (alpha MEM, GIBCO, Grand Island, NY). Cells were enumerated in a Coulter Counter (Coulter Electronics, Hialeah, Fla). As judged by nonspecific esterase staining, greater than 90% of the cells were monocytes. The cells were plated in 35 × 100-mm Falcon 3001 plastic Petri dishes (Falcon Labware, Oakland, Calif) at a density of 3.5×10^6 monocytes/mL. Cell viability was >90% as determined by trypan blue exclusion. Cell culture media consisted of alpha MEM supplemented with 10% autologous human serum, 15 mmol/L HEPES, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). After two hours of incubation at 37 °C in humidified 5% CO_2, the nonadherent cells and overlying media were removed and the plates washed three times with Hanks' balanced salt solution (HBSS). Fresh media containing variables to be examined were then added to the monolayer, and cells were incubated at 37 °C for three to seven days. Media were changed after three days of incubation.

U937 cells. U937 cells obtained from the tissue culture facility of the Lineberger Cancer Research Center (UNC-CH) were plated at 2×10^6 cells/mL in Dulbecco's modified MEM/Ham's F-12

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medium (GIBCO) that was supplemented with 5% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL).

Acid Phosphatase Determinations

Cytochemistry. Human monocytes were isolated as previously described, placed on tissue culture slides (LAB-TEK, Miles Scientific, Naperville, Ill), and incubated for the desired length of time. At the end of the incubation the media were removed. The slides were washed and air dried. U937 cells were suspended in culture media as previously described. At the end of the desired incubation period, cytopreparations were made of the cell suspensions by centrifugation at 1,500 rpm for five minutes (Shandon-Elliott Cytospin, Sewickley, Pa). Cytopreparations of U937 cells and adherent monocytes were air dried, fixed, and stained as described by Janckila et al.[4]

Colorimetric determination. Human monocytes were incubated the desired length of time, and the culture media were removed. Plates were washed twice with HBSS. The adherent monolayer was then disrupted with a rubber policeman in a 1-mL vol of 10 mmol/L N-tris[Hydroxymethyl]methyl 2-aminoethanesulfonic acid (TES) buffer. Cells disrupted by sonication and suspended in buffer were used in the biochemical assay. U937 cells were cultured for the desired period of time and washed three times in HBSS. The cell pellet was diluted to a concentration of 5×10^6 cells/mL in 10 mmol/L TES (pH 7.4). This suspension was sonicated for 30 seconds to form an extract. The extract was then centrifuged at 150 g for ten minutes. The supernatant was removed and used in the biochemical analysis. U937 cells treated with $1,25(OH)_2D_3$ required removal with a rubber policeman. The cells were then counted and prepared as described for the immature U937 cells.

Cell extracts (0.1 mL) were mixed with an assay mixture containing 9.2 mmol/L para-nitrophenylphosphate (P-NPP, Sigma) and 0.1 mol/L acetate buffer (pH 6) in a final volume of 0.60 mL. For the determination of TRAcP, the reaction mixture included 10 mmol/L L-sodium tartrate (Mallinkrodt, Inc). The reaction was stopped by the addition of 2.5 mL 0.05 mol/L NaOH. The color was measured at 410 nm in a Beckman Acta V spectrophotometer (Beckman Instruments, Inc, Fullerton, Calif). A Lowry protein assay was run simultaneously on cell extracts.[23] The amount of hydrolyzed P-NPP was then calculated by comparing the absorbance of the sample to a standard solution of P-NPP. Enzyme activity is defined as nanomoles of substrate hydrolyzed per minute (milliunits). L-ascorbic acid (10^{-3} mol/L) and Fe^{+2} (10^{-4} mol/L) were also added to some of the cell extracts containing L-sodium tartrate.

Isoenzymes determination. Human mononuclear phagocytes and U937 cells were cultured for the desired period of time. Media from phagocytes and U937 cells were removed and culture dishes washed vigorously with HBSS to remove residual serum. Cell extracts were analyzed by electrophoresis on an acidic acrylamide gel as previously described by Lam et al.[6]

RESULTS

Histochemistry of AcP Activity

Monocytes isolated from the peripheral blood of normal human volunteers were separated from lymphocytes by two

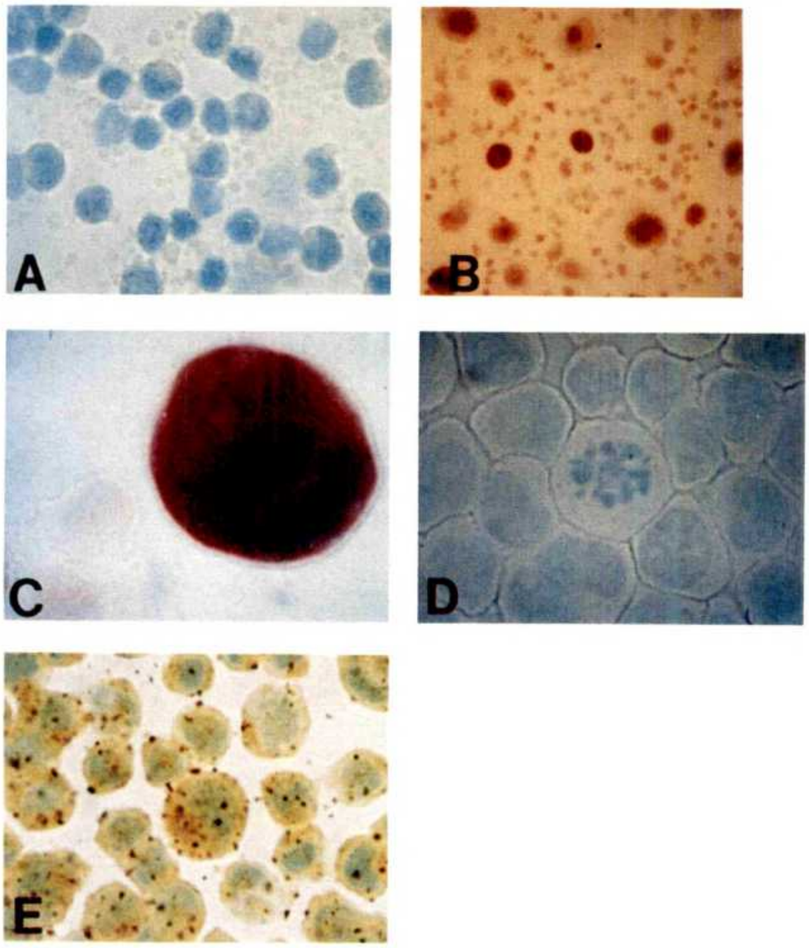

Fig 1. Light microscopic examination of phagocytic cells. TRAcP activity is represented by red stain. (A) Peripheral blood monocytes at two hours of culture have no tartrate-resistant activity (original magnification × 10). (B) After three days of culture, human monocytes demonstrate TRAcP activity. The activity is most intense in multinucleated giant cells (original magnification × 10). (C) Close-up of three-day giant cell with strong TRAcP activity (original magnification × 100). (D) Replicating U937 cells demonstrate no TRAcP activity (original magnification × 40). (E) In the presence of $1,25(OH)_2D_3$, a speckled pattern is seen that appears to represent extracellular enzyme activity (original magnification × 40).

hours of adherence and examined using standard staining technique for total and TRAcP activity.[4] As shown in Fig 1A, AcP expressed by these cells was inhibited by Na tartrate. After three days of incubation, monocytes developed the appearance of monocyte-derived macrophages,[22,24] and an increased number of multinucleated giant cells was noted. All cells demonstrated TRAcP activity, which was most intense in multinucleated giant cells (Fig 1B and C). Cells cultured over a seven-day period continued to express this activity.

We and others[18,19,25,26] have recently demonstrated a variety of effects of $1,25(\mathrm{OH})_2\mathrm{D}_3$ on the U937 cell line. This cell line also resorbs devitalized bone chips in vitro.[7] We examined the cytochemical AcP activity of this cell line under conditions similar to normal monocytes. Replicating (immature) U937 cells demonstrated tartrate-sensitive AcP activity (Fig 1D). Cells incubated for three days with $1,25(\mathrm{OH})_2\mathrm{D}_3$ (10^{-8} mol/L) showed increased maturation, as has been previously reported.[18,19] However, tartrate-sensitive AcP activity continued to be the primary enzyme expressed. A few cells demonstrated TRAcP activity in a speckled pattern near the cell membrane (Fig 1E).

Colorimetric Analysis

AcPs hydrolyze *p*-nitrophenol phosphate rapidly at a pH of 5 to 5.5. Peripheral blood monocytes demonstrated both tartrate-sensitive and tartrate-resistant AcP activity (Table 1). Monocytes in culture for three to seven days developed an increase in this activity (Table 1). Most of the activity was resistant to tartrate. Immature U937 cells demonstrated less AcP than human monocytes. U937 cells were differentiated by exposure to $1,25(\mathrm{OH})_2\mathrm{D}_3$, but only small differences were noted in AcP expression (Table 1). These results conflicted with the cytochemical examination and prompted further analysis of this enzyme activity.

Isoenzyme Expression of AcP

AcPs isolated from human tissues migrate as five distinct bands on gel electrophoresis, and these bands most likely represent distinct isoenzymes.[6,27] TRAcP can be localized to band 5, which migrates farthest toward the cathode. Band 5 appears as two components designated as 5a and 5b. They differ only by a carbohydrate moiety present on 5a, which can be removed by sialidase.[1] Band 5b can also be isolated from osteoclasts,[28] the neoplastic cells of leukemic reticuloendotheliosis,[13,14] and phagocytes of Gaucher's disease.[15-17] Freshly isolated monocytes studied by gel electrophoresis demonstrated only band 4 (tartrate-sensitive) activity, as previously reported (Fig 2).[22] Monocyte-derived macrophages cultured for three to seven days expressed band 5b (Fig 3). Monocytes incubated with $1,25(\mathrm{OH})_2\mathrm{D}_3$ also demonstrated band 5b on gel electrophoresis (data not shown).

Table 1. AcP Expression by Phagocytic Cells (mU P-NPP Hydrolyzed/mg Protein)

	AcP	TRAcP	Percentage of TRAcP
U937 cells (replicating)	9.6 ± .34	8.3 ± .3	86
U937 cells (differentiated)*	13.7 ± .36	10.6 ± .22	77
Monocytes	16.7 ± 5.9	13.9 ± 6.6	83
Monocyte-derived macrophages (three days in culture)	36.2 ± 3.8	25.3 ± 2.4	70

*Phagocytes were cultured for three days in the presence of 10^{-8} mol/L $1,25(\mathrm{OH})_2\mathrm{D}_3$.

Results are the mean ± SD of three to four separate experiments, each performed in duplicate.

In the U937 cell, only band 4 was detected, whether the cells were replicating or not. Band 4 activity was greater in the U937 cells incubated with $1,25(\mathrm{OH})_2\mathrm{D}_3$. Band 5b was not observed under these conditions (Fig 4). These results supported our cytochemical observations (Fig 1). To further evaluate the AcP activity of U937 cells, a cellular extract was eluted over a diethylaminoethyl (DEAE) cellulose column (Fig 5). Over 90% of the AcP activity eluted as a single peak that was tartrate sensitive and migrated on gel electrophoresis as band 4. Band 5b was not observed in this cell extract even after the addition of ferrous iron and ascorbic acid. These compounds are known to enhance the expression of the TRAcP.[29] The substance(s) responsible for the TRAcP activity observed in the colorimetric assay of the crude U937 extract and freshly isolated human monocytes has not been as yet identified.

DISCUSSION

TRAcP can be isolated in high concentration from bone.[9,28,29] Although the cell of origin of this enzyme has not

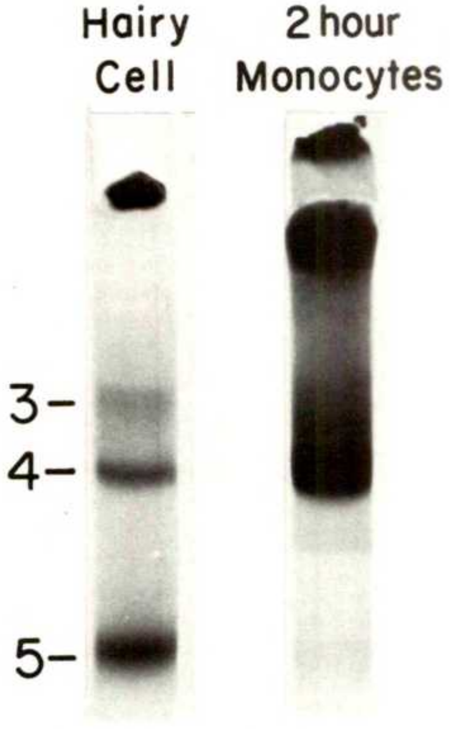

Fig 2. Gel electrophoresis of freshly isolated peripheral blood monocytes. Only band 4 activity is seen.

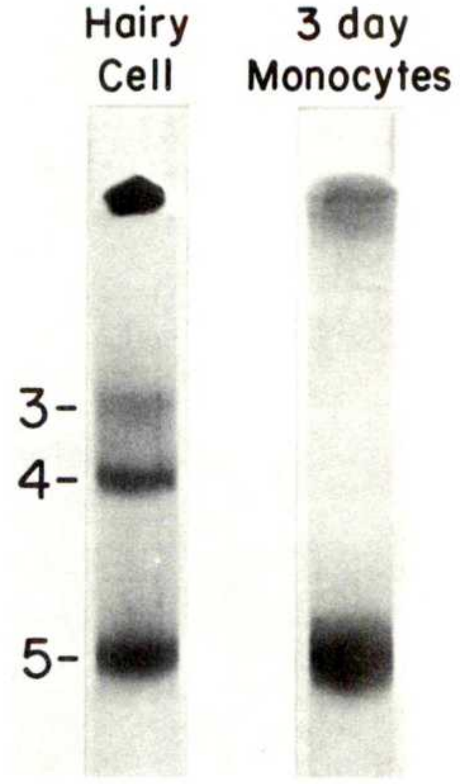

Fig 3. Gel electrophoresis of monocyte-derived macrophages (three days) in which band 5b activity is seen.

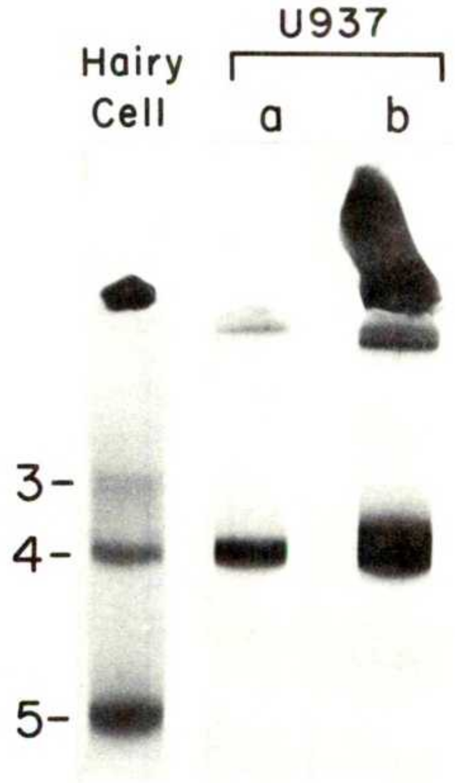

Fig 4. Gel electrophoresis of U937 cells: (A) Replicating cells have only band 4 activity. (B) In the presence of $1,25(OH)_2D_3$ band 4 activity is enhanced. Band 5 activity cannot be demonstrated.

been clearly defined, morphologic evidence suggests that TRAcP is associated with the osteoclast.[17] TRAcP is routinely used to identify osteoclasts isolated from bone or osteoclastlike cells in culture.[1,5,10] Several lines of evidence suggest that osteoclasts are derived from mononuclear phagocytes, although it is unclear whether peripheral blood monocytes[30,31] or stem cells[32,33] are required for such differentiation. In the present study we evaluated the AcP activity of peripheral blood monocytes, monocyte-derived macrophages, and the U937 cell line, a model for monocytic phagocytes.[21] Our methods included cytochemistry, colorimetric analysis, and gel electrophoresis. Cytochemical and colorimetric assays are widely used, and can be confirmed by gel electrophoresis[1,6] or immunoassay.[34]

Cytochemical analysis revealed only minimal TRAcP activity in freshly isolated normal human peripheral blood monocytes, a finding consistent with previous reports.[3,27,35] The strong TRAcP in cultured monocytes is consistent with recent observations by Weinberg and colleagues who demonstrated TRAcP activity in polykaryons formed from peripheral blood monocytes cultured in the presence of interferon-γ.[36]

U937 cells replicate in culture, remain in suspension, and are immature monocytes by several criteria.[21] These cells expressed only tartrate-sensitive cytochemical AcP activity. Cells incubated in the presence of $1,25(OH)_2D_3$, a known inducer of differentiation, occasionally expressed a speckled pattern of TRAcP activity. This pattern appeared to be located at the plasma membrane and may represent the absorption of the enzyme from serum in the culture medium.[8]

A colorimetric assay demonstrated TRAcP activity in both monocytes and U937 cells. This finding differed from the results derived from the cytochemical assay. To resolve this discrepancy, extracts from monocytes and U937 cells were evaluated by gel electrophoresis. Only band 4 of the

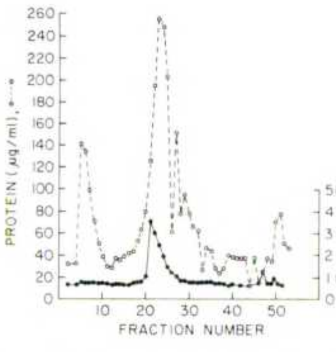

Fig 5. The U937 cell was subjected to sonication for one minute then centrifuged for 15 minutes at 20,000 g. The insoluble particulate fraction was discarded. The soluble proteins in the supernatant fraction were transferred to a DEAE-cellulose column (0.9 × 30 cm), and eluted by a linear concentration gradient of NaCl–10 mmol/L Tris-sulfate, pH 8. The eluate was analyzed for protein concentration and AcP activity by the colorimetric method. Most of the AcP activity was recovered in one peak corresponding to band 4 as detected by the electrophoresis method.

AcP isoenzymes was observed in the freshly isolated monocytes. Monocyte-derived macrophages expressed band 5b. The U937 cells showed only band 4, which increased after exposure to $1,25(OH)_2D_3$ Anderson and Toverud have previously reported that iron and ascorbic acid enhanced detection of TRAcP activity.[29] No band 5b was seen in the U937 cells, even in the presence of ferrous iron and ascorbic acid Ninety percent of the total AcP activity in the U937 cells was isolated chromatographically and identified as band 4.

These results have several important implications for the study of AcP. It seems clear that the various assays available for the detection of acid phosphatase do not uniformly agree. In our hands the cytochemical assay correlated best with the results of gel electrophoresis. Although the latter technique is relatively cumbersome, the results are more specific than for the colorimetric assays frequently used Phagocytic cells appear to possess substance(s) that interfere with use of the colorimetric identification of TRAcP.

Isoenzyme 5b was not observed in the U937 cell line even though it is an established monocytic cell model. Band 5b, an isoenzyme characteristic of osteoclasts,[28] was expressed in monocyte-derived macrophages. These findings demonstrate a biochemical link between the monocyte-derived macrophages and osteoclasts.

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Hydroxyurea Induction of Hemoglobin F Production in Sickle Cell Disease: Relationship Between Cytotoxicity and F Cell Production

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Initial alterations in fetal hemoglobin (HbF) production among eight sickle cell anemia subjects treated with hydroxyurea (Hu) are summarized. Four of these subjects had been previously treated with 5-azacytidine (5-aza). All subjects treated with Hu (50 mg/kg/d for three to five days) had suppression of their total reticulocyte counts by seven days, whereas the four subjects previously treated with 5-aza (2 mg/kg/d for three to five days) had increased reticulocyte counts at day 7. The effect of Hu on increasing the number of HbF-containing reticulocytes (F reticulocytes) is extremely variable, ranging from ten- to less than onefold differences in maximal posttherapy *v* pretherapy levels. Recovery from marrow suppression did not result in greater than twofold increases in F reticulocyte counts. Mean day 7 F reticulocyte levels in the four subjects treated with both Hu and 5-aza were 4.1 × 10/μL and 15.4 × 10^4/μL, respectively.

Among Hu-treated subjects, increased F reticulocyte production was correlated with low serum creatinine levels and rapid removal of Hu from the plasma. Furthermore, suppression of CFU-E colony formation on day 2 of therapy with Hu was inversely correlated with maximal F reticulocyte response. We conclude that where Hu treatment results in marrow toxicity (decreased reticulocyte counts, decreased CFU-E colony formation) HbF production is less likely to increase. Those sickle cell anemia subjects with minimal renal dysfunction (serum creatinine level, >1.0 mg/dL) exhibit the most cytotoxicity and least F reticulocyte response to Hu.

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INCREASED fetal hemoglobin (HbF) production may potentially decrease the clinical severity of sickle cell (SS) anemia.[1] Recently it was shown that 5-azacytidine (5-aza)[2-4] and hydroxyurea (Hu)[5] could increase HbF production in SS disease. The mechanisms by which these two agents increase HbF is unclear. Several investigations have suggested that these agents kill late erythroid precursors (CFU-E), leading to recruitment from earlier progenitors (BFU-E) that have retained their HbF-producing capacity.[6-8] In previous reports we have demonstrated that 5-aza rapidly increases HbF production without marrow toxicity[9] and without reduction in CFU-E colony formation.[10]

In this report we summarize initial responses to Hu therapy in four SS subjects who previously responded to 5-aza and report observations on four additional subjects who have been given Hu only. Unlike 5-aza therapy, which causes little if any toxicity in the first seven days of therapy, Hu therapy leads to early suppression of the reticulocyte count and in some patients to decreased CFU-E colony growth. Marked variation in the ability to increase HbF production was seen in the Hu-treated patients. Increased HbF production was observed only in those patients with rapid decrease of Hu from their plasma and with the least suppression in CFU-E and BFU-E colony formation.

MATERIALS AND METHODS

Subject selection All subjects studied were adults with SS disease followed at Johns Hopkins Hospital (subjects A, C, and E) or the National Institutes of Health (NIH) (subjects 6, 7, 10, 11, and 12). The effects of an optimal dose of 5-aza (1.5 to 2 mg/kg/d) on subjects A, C, 6, and 7 have been previously reported [4,9,10] Subjects E, 10, 11, and 12 were given only Hu. The dose of Hu, 50 mg/kg/d for three or five days, was chosen on the basis of previous reports in animals and man.[5,6] Subjects 6 and 7 were given an additional single dose of Hu (25 mg/kg) and followed for seven days All subjects gave informed consent for these experiments under the guidelines outlined by the Joint Committee on Clinical Investigation at Johns Hopkins Hospital or the National Heart, Lung, and Blood Institute Review Board

HbF production Previously described radial immunoprecipitation assays were used to estimate the percentage of HbF-containing reticulocytes (F reticulocytes).[11] The red cell number was enumerated in a Coulter Counter (Coulter, Hialeah, Fla), and the percentage of total reticulocytes was estimated after staining with new methylene blue.

Hu levels. Plasma Hu levels were analyzed by the method of Fabricius and Rajewsky.[12] The assay is a colorimetric procedure based on the oxidation of Hu, with iodine forming nitrite that diazotizes sulfanilic acid. The diazotized sulfanilic acid is then coupled with *N*-(1-napthyl)-ethylenediamine dihydrochloride to produce a red color Samples were separated from whole blood, frozen at −20 °C and assayed within two days of collection. Serum creatinine levels were determined at least five times prior to and during experimental therapy by routine clinical methods

In vitro analysis Assays of early and late erythroid progenitors (BFU-E and CFU-E) were performed as previously described[10] in cultures that contained 0.8% methylcellulose (Dow Chemical Co, Midland Mich), 30% fetal calf serum (Flow Laboratories, McLean, Va), 2.5 μ/mL of erythropoietin (Connaught Laboratories, Willowdale, Canada), and 10% phytohemagglutinin-stimulated leukocyte-conditioned media.

RESULTS

F reticulocyte response. Figure 1 details the F reticulocyte response of eight SS subjects given Hu A greater than twofold increase in the F reticulocyte occurred in only four subjects (10, 12, A, E). Figure 2A illustrates that all eight subjects showed a decrease in total reticulocytes after seven days of Hu therapy. In contrast, the four subjects previously

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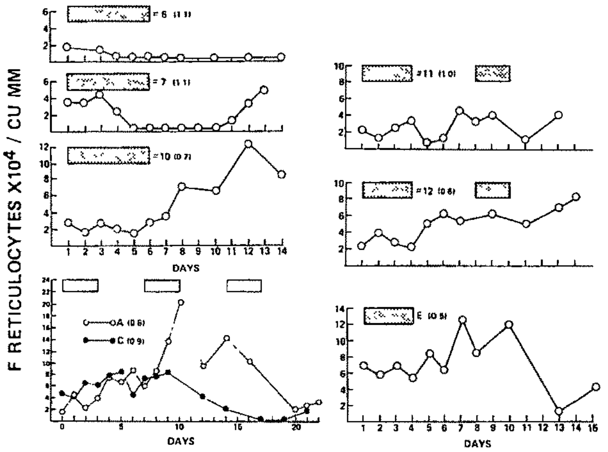

Fig 1. F reticulocyte response to Hu therapy. Each subject was treated with 50 mg/kg/d (shaded boxes). The number in parentheses refers to the serum creatinine level of each subject.

treated with 5-aza (A, C, 6, 7) showed no evidence of reticulocyte suppression at seven days following 5-aza therapy. Reticulocyte levels returned to pretreatment levels within seven days of their nadir in all Hu-treated subjects except subject 6. Subject 6 required a transfusion on day 15, after the hemoglobin level dropped from 9.8 to 5.0 g/dL following Hu administration. F reticulocyte levels increased with recovery from marrow suppression in subject 7, but the maximal F reticulocyte response (day 13) was not greater than twofold the pretreatment levels.

In all eight subjects given Hu, day 7 F reticulocyte levels were lower than those attained in the four subjects given 5-aza (Fig 2B). The mean day 7 F reticulocyte level for the four subjects given 5-aza and Hu were 15.4 × $10^4/\mu L$ and 4.1 × $10^4/\mu L$, respectively

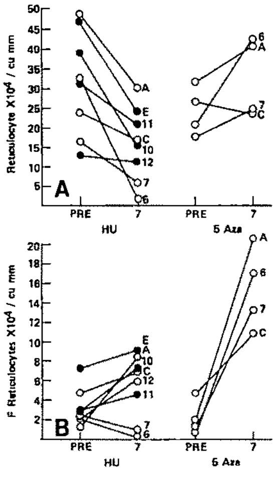

Fig 2. Comparison of the effect of Hu and 5-aza on (A) the total reticulocyte count and (B) the F reticulocyte count. Pretherapy (PRE) and day 7 (7) values are given. Open circles indicate subjects treated with both drugs. Closed circles are subjects treated only with Hu. Numbers and letters are the same as listed in Fig 1.

The two subjects who exhibited the most toxicity (6 and 7) were given a lower dose of Hu (25 mg/kg) and followed for seven days. In subject 6 the total reticulocyte count decreased slightly (41 to 34 × $10^4/\mu L$), and the F reticulocyte count did not change (1.5 to 1.6 × $10^4/\mu L$). Subject 7's total reticulocyte count also decreased (32 to 24 × $10^4/\mu L$), but his F reticulocyte count increased (3.8 to 7.0 × $10^4/\mu L$).

Hu levels. Normally more than 80% of Hu is excreted within six hours in the urine.[13,14] Plasma Hu levels were measured following a standard oral dose of 25 mg/kg of Hu in all eight subjects. Plasma levels of Hu are shown for four subjects in Fig 3. Subjects 6 and 7 who showed little if any F reticulocyte response and a marked reduction in total reticulocytes had delayed plasma Hu clearance. Subjects A and 12 had normal plasma clearances of Hu, less severe suppression in total reticulocytes (Fig 2), and ten- and fourfold increases in the F reticulocyte level.

Minimal renal dysfunction might prolong clearance of Hu from the plasma and thereby lead to increased renal toxicity. Mean serum creatinine levels during therapy for each subject (range, 0.5 to 1.1) correlated significantly with six-hour plasma Hu levels, $r = .85$, $P = .01$. Moreover, the six-hour level of Hu and the mean serum creatinine levels were negatively correlated with maximal F reticulocyte responses ($r = .80$, $P = .03$; and $r = .70$, $P = .05$).

In vitro analysis. Bone marrow–derived CFU-E colonies were assayed in all five subjects studied at the NIH (Table 1). The F reticulocyte response as measured by the ratio of maximal F reticulocyte levels to pretreatment levels was inversely correlated with the suppression of CFU-E colony growth at two days of therapy. Subject 6, who had the most profound reticulocyte suppression (Fig 2), showed the greatest reduction in CFU-E on day 2. An analysis of data regarding BFU-E colony growth suggests that suppression may also be related to the F reticulocyte response. Subject 10, who showed the highest F reticulocyte response, had no suppression of BFU-E colonies at day 2. Subject 12, who showed an intermediate response, had an early suppression of BFU-E (19%, day 2), but some later recovery (80%, day 7). Subjects 11 and 7, who showed relatively low F reticulocyte responses, showed profound suppression of BFU-E colonies on day 2 and day 7.

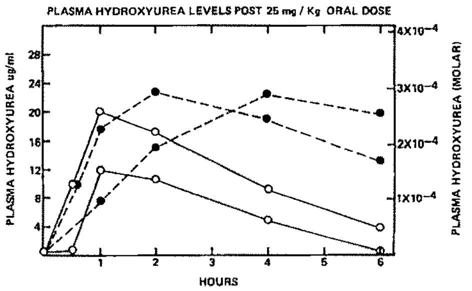

Fig 3 Plasma Hu levels measured following an oral dose of 25 mg/kg Hu. Open circles represent subjects A (peak level, 20 μg/mL) and 12 (peak level, 12 μg/mL), and closed circles represent subjects 6 (peak at four hours, 22 μg/mL) and 7 (peak at two hours, 23 μg/mL).

Table 1. Comparison of F Reticulocyte Increments and CFU-E Colony Formation During Hu Therapy

Subjects	F Reticulocyte Ratio* (Post/Pre)	CFU-E† (%)		BFU-E‡ (%)	
		Day 2	Day 7	Day 2	Day 7
10	4.6	103	—	160	—
12	3.6	82	82	19	80
11	2.1	22	8	26	4
7	1.3	23	6	0	0
6	0.1	2	—	—	—

*Post = maximum F reticulocytes × $10^4/\mu L$ (See Fig 1). Pre = pre-F reticulocytes × $10^4/\mu L$.

†Number of CFU-E colonies expressed as a percentage of day 0 (pretherapy) CFU-E colonies The pretherapy number of CFU-E colonies/10^5 nucleated cells were 107, 78, 314, 60, and 85 for subject 10, 12, 11, 7, and 6, respectively

‡Number of BFU-E colonies expressed as a percentage of day 0 (pretherapy) BFU-E colonies. The pretherapy number of BFU-E colonies/10^5 nucleated cells were 38, 57, 152, and 50 for subjects 10, 12, 11, and 7, respectively

DISCUSSION

In a previous report by Platt et al,[6] two SS subjects given Hu, 50 mg/kg/d divided into three doses for five days, increased F reticulocyte levels without significant suppression of reticulocytes. In this report, eight subjects treated with comparable doses for three to five days showed suppression of total reticulocyte counts, with marked suppression seen in two of our subjects. The number of F reticulocytes after seven days of therapy with Hu increased slightly in six out of eight subjects even though all subjects experienced suppression in total reticulocytes. Even after recovery of the marrow, F reticulocyte levels were not significantly increased. Furthermore, the rapid increase in F reticulocytes (two to three days after starting therapy) previously reported[3,9,10] following 5-aza therapy is not seen in our subjects treated with Hu.

Our subjects showed considerable differences in their F reticulocyte responses to Hu. A clue to the origin of that variability is apparent when serum creatinine and plasma Hu levels are analyzed Minimal renal dysfunction leads to delayed clearance of Hu from the plasma. Previous investigators have shown that peak plasma levels of Hu $>10^{-4}$ mol/L (7.6 μg/mL) will cause marrow cytotoxicity in man.[14] The peak levels obtained in all subjects given a 25-mg/kg test dose of Hu are in the cytotoxic range. In subjects with higher serum creatinine levels (Fig 3), plasma Hu levels remain elevated for a longer period of time, leading to significant marrow suppression and decreased F reticulocyte response.

The differences in the Hu-induced F response of the subjects reported herein and those of Platt et al[5] are unclear. Presumably, the peak drug level after a single 50 mg/kg dose given to our subjects would be much higher and possibly more toxic than the divided doses given by Platt et al. However, when the two subjects (6 and 7) with the greatest toxicity to a single dose of 50 mg/kg/d were given lower doses, both showed suppression of total reticulocyte count levels, and only one showed a marginal increase in F reticulocytes. The two subjects treated by Platt et al were younger than our eight subjects, ages 17 and 23 v ages ranging from 27 to 44 in our subjects. Both of Platt's subjects had normal serum creatine levels, 0.3 and 0.5 (personal communication, Platt). Our data would suggest that these low serum creatine values would favor a higher F reticulocyte response. Finally, preliminary information on two subjects (A and C) with serum creatinine values of 0.6 and 0.9 subsequently treated at Hopkins suggests that less cytoxicity and a more sustained F response may be achieved if the amount of Hu given three days per week is reduced to 20 to 40 mg/kg/d.

This report also indicates that drugs given at doses that caused detectable marrow suppression are unlikely to be of benefit in SS subjects since the result of such suppression is a decrease in F cell production. These observations are important since others[6-8] have suggested that the mechanism of action of 5-aza and Hu is the selective cytotoxic effect on late erythroid progenitors (CFU-E) and subsequent recruitment of high HbF-producing early erythroid progenitors (BFU-E). In a previous report[10] we have shown that CFU-E suppression is not associated with increased F reticulocyte response with 5-aza. In this report we show that patients who had the least measured toxicity to their CFU-E on day 2 of treatment (subjects 10 and 12) had the highest F reticulocyte response. Conversely, the subjects with the most profound decrease in CFU-E at two days of therapy were the ones with the least F reticulocyte response These data along with previous information[10] that shows no cytotoxicity of CFU-Es following 5-aza therapy suggest that selective CFU-E cytotoxicity is not necessary for the early increases in F reticulocytes following 5-aza or Hu.

It is unclear whether 5-aza and Hu increase F cell production in the same way. In those subjects treated with both drugs, the F reticulocyte response is more rapid and consistently higher with 5-aza than with Hu. 5-aza increased F cell production without suppression of CFU-E on day 2 of therapy. In this report the highest response among the patients studied at the NIH was the individual with no detectable suppression of BFU-E or CFU-E. Clearly, cytotoxicity of erythroid precursors is not necessary for a rapid increase in F cell production. Some increase in F cell production, however, may result from drug therapies that selectively inhibit CFU-E and not BFU-E. Unfortunately, our data suggest that Hu treatment is more likely to suppress both CFU-E and BFU-E. Careful adjustment of Hu dosage based on measured plasma levels after a test dose may permit a more selective effect.

Since selective cytoreduction of late erythroid precursors (CFU-E and pronormoblasts) is not associated with increased HbF productions in clinical trials using 5-aza and Hu,[3,5,10] alternative mechanisms must now be considered. Other agents (vincristine, cytosine arabinoside) that perturb cell-cycle kinetics have been associated with increased HbF production.[8,15] We now favor the hypothesis that perturbation of the cell cycle without cell death may in some way change the regulatory patterns that control fetal and adult hemoglobin production. No information is available as yet that indicates how perturbation of the cell cycle might alter gene expression. Perhaps reversible delays in cell division lead to the accumulation of unstable proteins. These proteins may act as trans-acting substances that modulate differential globin gene expression. Further tests are now underway to test several aspects of this general hypothesis.

Neither this study nor previous reports of 5-aza[2,4,9] and Hu[5] indicate that these drugs have therapeutic benefit in SS disease. Further studies are necessary to determine whether lower or less frequent doses of Hu will result in greater F reticulocyte response and less cytotoxicity A previous uncontrolled study[9] showed that prolonged 5-aza therapy can decrease anemia and may decrease the severity and frequency of vaso-occlusive crisis Controlled clinical trials are necessary to determine the efficacy of 5-aza or Hu therapy, but it is unlikely that such trials can be carried out until the carcinogenicity of the two drugs can be determined more completely.[16,17] This report indicates that Hu is also more difficult to use because of the apparently narrow window between cytotoxicity and the desired effect on increased HbF production.

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Diffuse Large Cell Lymphoma in a Patient With Hairy Cell Leukemia: Immunoglobulin Gene Analysis Reveals Separate Clonal Origins

By James R. Downing, Carlo E. Grossi, Carl T. Smedberg, and Peter D. Burrows

A 75-year-old man with hairy cell leukemia (HCL) was found to have an immunoblastic lymphoma of the small bowel. Immunologic and genotypic characterization of these neoplasms revealed both the HCL and the immunoblastic lymphoma to be of the B cell lineage. The HCL expressed the HCL-associated antigens detected by the monoclonal antibodies HC-1 and HC-2, whereas the immunoblastic lymphoma failed to react with these antibodies. In addition, surface immunoglobulin light chains could not be accurately determined for the hairy cells, whereas the immunoblastic lymphoma was shown to express only κ immunoglobulin light chains. These immunophenotype differences were compatible with either the clonal evolution of the HCL into the immunoblastic lymphoma or a separate clonal origin for these two neoplasms. An analysis of tumor DNA by Southern blot hybridization revealed different heavy-chain and κ light-chain gene rearrangements in these two malignancies. Thus the occurrence of the large cell lymphoma most likely represents the emergence of a second clonally unrelated B cell malignancy.

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HAIRY CELL LEUKEMIA (HCL) is a chronic low-grade malignant lymphoproliferative disease characterized by pancytopenia, splenomegaly, and morphologically distinct lymphocytes in the bone marrow, spleen, and occasionally the blood.[1-4] Considerable controversy has existed over the lineage of the neoplastic cells of HCL.[5-16] However, recent analyses of the immunoglobulin genes in 19 cases of HCL have revealed rearrangements of a heavy-chain gene locus and at least one light-chain gene locus in each case, confirming the B cell nature of this lymphoproliferative disorder.[17,18] Unlike other chronic B cell neoplasms, HCL rarely shows histologic progression to a high-grade non-Hodgkin's morphology Occasional instances of the coexistence of HCL and diffuse histiocytic lymphoma have been reported.[19-22] It has been suggested that this coexistence represents the histologic progression or clonal evolution of the hairy cell leukemia into the diffuse histiocytic lymphoma, although little evidence in support of this contention has been presented. In this report we present the detailed immunologic and molecular genetic analysis of a patient with HCL who early in his course was found to have a large cell immunoblastic lymphoma. Our results suggest that the immunoblastic lymphoma represents the emergence of a second malignant B cell clone differing from the original B cell clone of the HCL

CASE REPORT

The patient, a 75-year-old man, had a nine-month history of refractory anemia. A bone marrow biopsy performed in January 1984 was interpreted as representing refractory anemia with excess blasts. The patient was treated with oxymethalone 100 mg daily and prednisone 60 mg daily for 6 weeks with little response In June 1984 he was referred to the University of Alabama Hospitals with general malaise, easy fatigability, abdominal pain, fevers, and a productive cough. On physical examination he was afebrile and mildly cachectic with hepatosplenomegaly and right upper lobe crackles. Laboratory values included a hemogloblin level of 9.7 mg/dL; a platelet count of 94,000/mm³; a white blood cell count of 800/mm³, of which 20% were small lymphocytes; and a serum bilirubin level of 2.1 mg/dL. Chest x-ray demonstrated a right upper lobe infiltrate. A bone marrow biopsy showed a diffuse infiltration of small mononuclear cells along with myeloid hyperplasia and immaturity. This biopsy was again interpreted as representing a myelodysplastic condition, and the patient was successfully treated for his pneumonia with Mezlocillin, Tobramycin, and Erythromycin. Four months later the patient was readmitted for progressive general malaise, easy fatigability, lower abdominal pain, fevers, an 8-pound weight loss, and progressive splenomegaly. Laboratory values revealed a hemoglobin level of 10.8 mg/dL; a platelet count of 37,000/mm³, and a white blood cell count of 800/mm³, of which 83% were small lymphocytes. A bone marrow biopsy showed a diffuse infiltration of small lymphoid cells and was diagnosed as HCL. The patient underwent a therapeutic splenectomy in late October 1984 The resected spleen weighed 2,025 g and showed histologic features consistent with involvement by HCL. At surgery a 5-cm mass was discovered in the jejunum with extension into the mesentery, and this was resected. Histologically, the small-bowel lesion showed infiltration by large pleomorphic lymphocytes consistent with a non-Hodgkin's lymphoma of high-grade malignancy (diffuse histiocytic lymphoma, Rappaport Classification; malignant lymphoma, large cell, immunoblastic, Working Formulation). The patient was treated with Cytoxan 697 mg, Adriamycin 46 mg, Oncovin 1 mg, and prednisone 50 mg daily for five days. Despite three subsequent courses of chemotherapy, hepatomegaly and pancytopenia with circulating hairy cells persist

MATERIALS AND METHODS

Morphology and cytochemistry Portions of the biopsy tissues obtained after appropriate informed consent were fixed in phosphate-buffered formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin (H & E) Peripheral blood smears were stained with Wright-Giemsa stain. Bone marrow aspirates were stained with Wright-Giemsa stain, periodic acid-Schiffs reagent, or acid phosphatase by standard techniques.[23] The tartrate-resistent acid phosphatase (TRAP) activity was determined by the addition of a tartaric acetate buffer to the incubation mixture [24]

Immunologic characterization Mononuclear cells were isolated from heparinized blood samples by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation Cell suspensions from bone marrow aspirate and bowel tissue were obtained by gentle homogenization and subsequently freed of erythrocytes, mature granulocytes, and dead cells by Ficoll-Hypaque density gradient centrifugation

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Surface marker expression was determined on viable cells using mouse monoclonal antibodies followed by fluorescein isothiocyanate–labeled goat antimouse immunoglobulin (Southern Biotechnology Associates, Birmingham, Ala.). Positive cells were evaluated by both fluorescence microscopy and flow cytometry (FACS-Analyzer, Becton-Dickinson, Sunnyvale, Calif).

Anti–T cell antibodies of the Leu series and anti-HLA-DR were provided by Becton-Dickinson (Mountain View, Calif). The pan-B monoclonal antibody B-1 was obtained from Coulter (Hialeah, Fla), and the anti-immunoglobulin reagents with specificities for human light or heavy chains were purchased from Southern Biotechnology Associates. Monoclonal antibodies HC-1 and HC-2 with specificities for hairy cells were the kind gift of Dr David Posnett (Rockefeller University, New York)[25]

Gene rearrangement studies High molecular weight DNA from peripheral blood mononuclear cells or from fresh cell suspensions prepared from the small-bowel mesenteric mass was isolated according to standard procedures.[26] Twenty micrograms of the DNA was digested (3U/μg) with the restriction endonucleases *Bam*HI or *Eco*RI (IBI, New Haven, Conn) and size-fractionated on 0 7% agarose gels The DNA fragments were transferred out of the gel to Zetabind® filters (AMF, Cuno, Meriden, Conn) as described by Southern.[27] Filters were hybridized with nick-translated ^{32}P-radiolabeled human immunoglobulin gene DNA fragments with specific activities of 1×10^8 cpm/μg DNA. The probe for the human heavy-chain locus consisted of a 6 5-kb *Hin*dIII/*Bam*HI genomic J_H insert. This probe recognizes rearrangements in either *Bam*HI- or *Eco*RI-digested DNA. Rearrangements of κ genes were detected within *Bam*HI-digested DNA by using a 2.5-kb genomic *Eco*RI $C\kappa$ probe. Rearrangements of λ genes were detected within *Eco*RI-digested DNA by the combined 7 6-kb genomic *Eco*RI C_λ (Mcg) fragment and a 6 0-kb genomic *Eco*RI C_λ (Ke$^-$Oz$^-$) fragment. The structure of these probes is fully described elsewhere.[28-30] Hybridization reactions were carried out using 50% formamide and 10% dextran sulfate at 42°C. After extensive washing at the appropriate stringency, the filters were exposed to Kodak XAR-5 film using a single intensifying screen for 48 to 144 hours at −70°C.

RESULTS

Morphology and cytochemistry. Sections of the initial bone marrow biopsy revealed an increase in immature mononuclear cells of undetermined lineage along with myeloid and megakaryocytic hyperplasia. Although not diagnostic, the distribution of the mononuclear cells and their cytologic features were in retrospect suggestive of HCL. All subsequent bone marrow biopsies are in retrospect diagnostic of involvement by hairy cell leukemia. In these biopsies the cellularity was 80%. There was a diffuse infiltration of the marrow by small mononuclear cells having irregular bean-shaped nuclei, irregularly clumped chromatin, and a large rim of pale eosinophilic cytoplasm frequently showing retraction artifacts. Mitoses were extremely rare. Occasional clusters of normal maturing myeloid and erythroid elements were identifiable. The mononuclear cells in the bone marrow aspirate had the typical morphology of hairy cells with abundant basophilic cytoplasm, marked irregularity of the cytoplasmic borders, and small central hyperchromatic nuclei. Acid phosphatase stains of the bone marrow aspirate revealed positively staining intracytoplasmic granules in all of the mononuclear cells with no loss in acid phosphatase activity after exposure to tartrate.

The spleen weighed 2,025 g. The cut surface was firm with a focal micronodular texture. Histologically, the red pulp of the spleen was infiltrated by mononuclear cells similar to those seen in the bone marrow (Fig 1). The cells had abundant pale cytoplasm and small irregular nuclei and were frequently separated from each other by clear spaces. This mononuclear infiltrate focally extended into the white pulp. Numerous small blood lakes typical of HCL were identified within the red pulp of the spleen. At the time of splenectomy, a peripheral blood smear showed 20% lymphocytes that had the cytologic features of hairy cells.

A 5-cm segment of jejunum was removed during laparotomy There was marked thickening of the bowel wall with loss of the normal mucosal folds and marked mucosal thinning. The bowel wall was infiltrated by large pleomorphic mononuclear cells with large irregular nuclei, peripheral condensation of the chromatin, prominent eosinophilic nucleoli, and abundant amphophilic cytoplasm (Fig 2). Numerous mitotic figures were identified along with extensive single cell necrosis. The histologic features of this bowel lesion were diagnostic of a diffuse large cell non-Hodgkin's lymphoma (malignant lymphoma, large cell, immunoblastic, Working Formulation).

Immunologic characterization. Data on the expression of immunoglobulin products and other lymphocyte surface markers are summarized in Table 1. Approximately 20% of the cells in the peripheral blood and bone marrow aspirate were leukemic. The leukemic cells in these samples expressed the B-1 and HLA-DR antigens as well as the HCL-associated antigens detected by the monoclonal antibodies HC-1 and HC-2.[25] Analysis of surface immunoglobulin expression on peripheral blood mononuclear cells was equivocal with both κ- and λ-bearing lymphocytes present. A restricted expression of the γ heavy-chain isotype was detected, suggesting a probable B cell clone. The lymphoma cells from the small-bowel mass were shown to consist of a monoclonal B cell population that expressed surface μ and δ heavy chains and κ light chains. These cells were negative for antigens detected by the monoclonal antibodies HC-1 and HC-2.

Gene rearrangement studies. Studies of the arrangement of the immunoglobin heavy- and light-chain genes were performed on peripheral blood mononuclear cells (PBMC), and a mononuclear cell suspension prepared from the small-bowel mass. Utilizing *Bam*HI-digested DNA and a J_H probe, a single but different clonal rearrangement of the immunoglobulin heavy-chain gene locus was detected in each sample (blood, 19 kb; bowel, 11 kb; Fig 3). The intensity of the clonally rearranged band detected in the PBMC was less than the germ-line band and is consistent with a leukemic clone comprising a minority of the total PBMC. Analysis of the κ immunoglobulin light-chain locus within *Bam*HI-digested DNA utilizing a C_κ probe also revealed a single but different clonal rearrangement in each sample (blood, 6 kb; bowel, 18 kb; Fig 3). The λ light-chain gene locus as analyzed within *Eco*RI-digested DNA was in the germ-line configuration in both samples (Fig 3). These results indicate that although the expressed light-chain isotype within these two B cell malignancies are the same, the immunoglobulins are

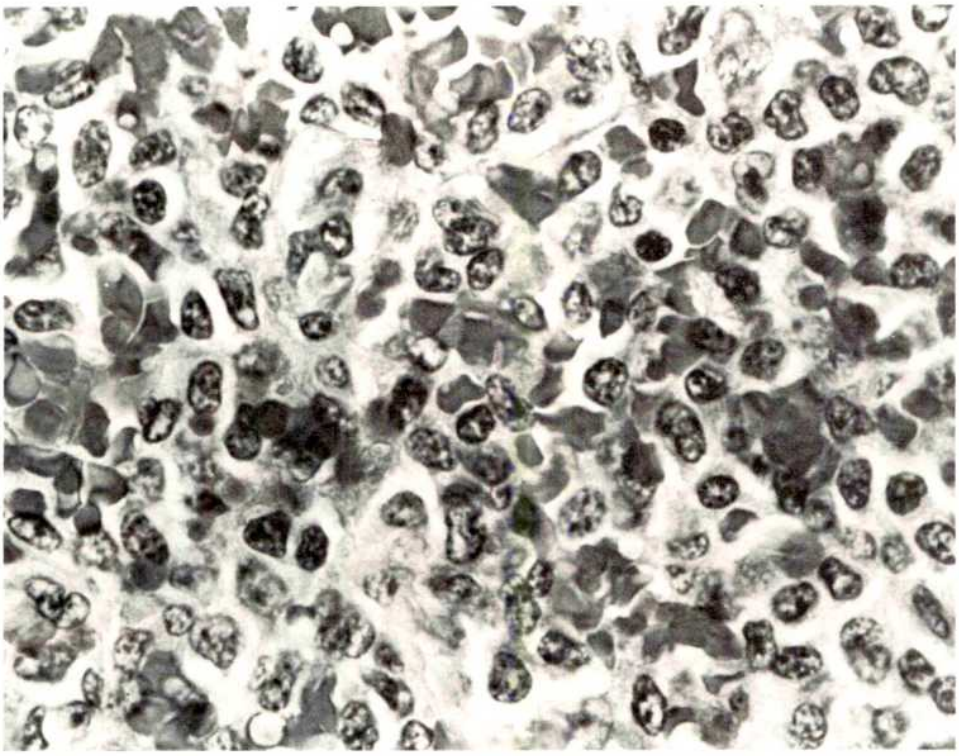

Fig 1. Splenic red pulp showing infiltration by leukemic hairy cells. The cells have small irregular nuclei and moderate clear cytoplasm with indistinct cytoplasmic borders. (H&E, original magnification ×1000; current magnification ×850)

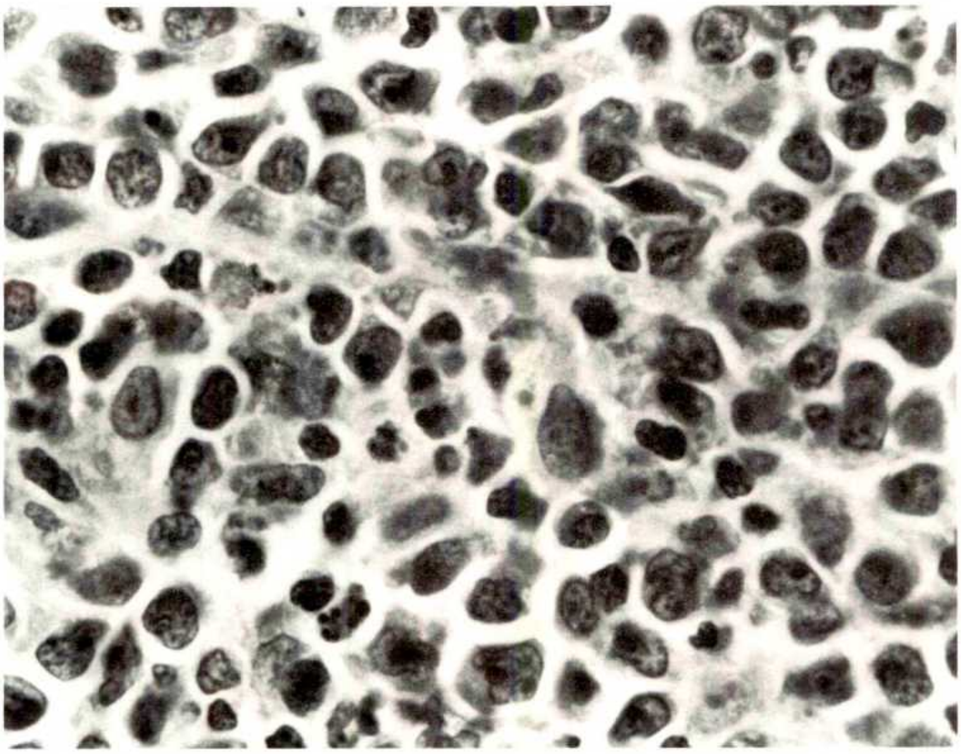

Fig 2. Small-bowel specimen showing infiltration by pleomorphic lymphoid cells having large round nuclei, peripheral condensation of the nuclear chromatin, prominent nucleoli, and a moderate amount of cytoplasm. Numerous mitotic figures are present as well as single-cell necrosis. The histologic features are diagnostic of immunoblastic lymphoma (H&E, original magnification ×1000; current magnification ×850)

Table 1. Cell Surface Markers

	Monoclonal Antibodies*							Surface Immunoglobulin†					
Sample	Leu 4	Leu 3	Leu 2	B-1	HLA-DR	HC-1	HC-2	μ	δ	γ	α	λ	κ
Blood	62	35	24	23	28	21	23	−	−	+	−	+	+
Bone marrow	ND	ND	ND	17	26	21	37	ND	ND	ND	ND	ND	ND
Bowel	16	5	4	68	41	0	0	+	+	−	−	−	+

ND, not determined.

*Percentage of reactive cells by immunofluorescent antibody determination.

†Surface immunoglobulin expression for leukemic hairy cells and lymphoma cells.

encoded by differently rearranged heavy- and light-chain genes.

DISCUSSION

This patient had the typical clinical and histopathologic features of HCL. The leukemic cells in both blood and bone marrow expressed B cell–associated antigens and the hairy cell–associated antigens detected by the monoclonal antibodies HC-1 and HC-2. In addition, mononuclear cells from bone marrow were positive for tartrate-resistant acid phosphatase. The B cell nature of this malignancy was confirmed by the presence of clonal rearrangements of the immunoglobulin heavy- and light-chain gene loci, a finding consistent with results from two recent studies on HCL.[17,18]

Although the occurrence of a second malignancy in patients with HCL is not uncommon, the occurrence of a high-grade non-Hodgkin's lymphoma as described in this case is extremely rare.[20,31-33] Several case reports of patients with HCL and large cell lymphoma have been published.[19-22] In each case the diagnosis of large cell lymphoma was based primarily on the interpretation of histologic material. Several of these reports suggest that the large cell lymphoma represented a blastic transformation of the hairy cells, a situation similar to the Richter transformation of chronic lymphocytic leukemia.[34,35] Davis et al[22] have recently described a case of HCL and large cell lymphoma in which both malignancies expressed tartrate-resistant acid phosphatase. Based on these data, the authors suggested that the large cell lymphoma represented the clonal evolution or blastic transformation of the original HCL. Thus, although supportive data for the idea of the clonal evolution of hairy cell leukemia into a high-grade lymphoid malignancy exist, no detailed analysis of the relationship of the malignant clones of the low- and high-grade tumors is presently available.

In this study we performed immunophenotyping and molecular genetic analysis of the malignant clones in a patient with HCL and immunoblastic lymphoma. The immunologic and genetic characterization of these two malignancies demonstrated phenotypes and genotypes that were consistent with B cell HCL and B immunoblastic lymphoma. Although both malignancies were of the B cell lineage, immunophenotypic differences between these malignant clones existed. The absence of the antigens detected by HC-1 and HC-2 on the immunoblastic lymphoma and the clear ability to detect its surface immunoglobulin phenotype could be compatible with the clonal evolution of the HCL into immunoblastic lymphoma. If this had been the case, both the HCL and the immunoblastic lymphoma cells would have had identical heavy- and light-chain immunoglobulin gene rearrangements. However, Southern blot hybridization analyses revealed different heavy- and light-chain gene rearrangements in these two malignancies (Fig 3). These results are consistent with the large cell lymphoma representing the emergence of a second B cell malignancy. The possibility that these two B cell malignancies are related at the stage of a common premalignant B cell precursor cannot be excluded from these data.

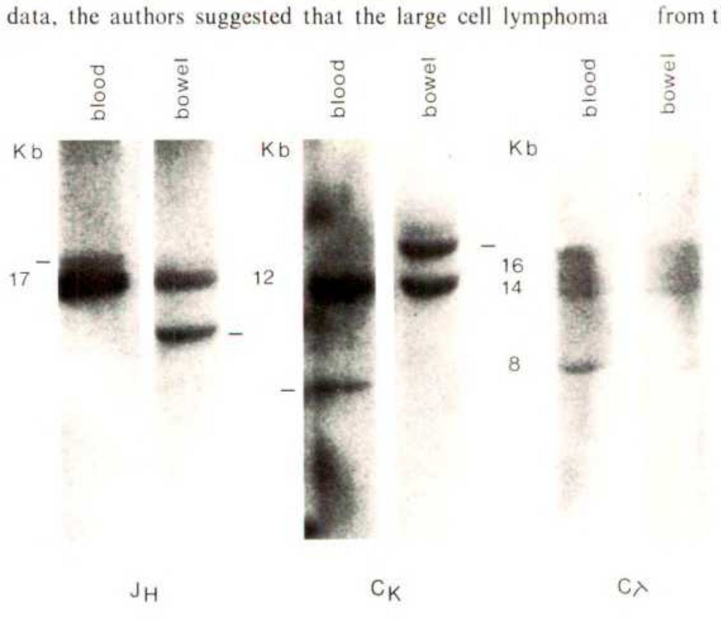

Fig 3. Southern blot analysis of immunoglobulin genes in peripheral blood mononuclear cells ("blood") and small-bowel mononuclear infiltrate ("bowel"). For the heavy-chain (J_H) and κ light-chain (C_κ) gene analysis, the DNA was cleaved with the restriction endonuclease *Bam*HI. For λ light-chain (C_λ) gene analysis, the DNA was cleaved with the restriction endonuclease *Eco*RI. Germ-line bands are indicated by their corresponding size in kilobases (kb). Lines indicate the position of rearranged bands.

The finding that the so-called blastic transformation or histologic progression of a low-grade lymphoproliferative lesion in fact represents the emergence of a second tumor is not without precedent. A recent study on a patient with Richter's transformation revealed that the high-grade pleomorphic tumor and the chronic lymphocytic leukemia expressed different light-chain isotypes and had different heavy- and light-chain gene rearrangements.[36] In addition, an examination of immunoglobulin gene rearrangements in seven cases of low-grade non-Hodgkin's lymphoma that showed histologic progression on a second biopsy revealed that in three cases the high-grade tumor represented the emergence of a new, genetically distinct clone.[37] Our findings in conjunction with these suggest that the occurrence of a second B cell malignancy in a patient with a preexisting B cell tumor may be a more frequent event than is presently appreciated. Detailed immunophenotyping and molecular genetic analysis of additional cases of patients with HCL and lymphoma will be needed to determine if HCL can ever show true clonal evolution or blastic transformation into a high-grade tumor.

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Carbonyl Iron Therapy for Iron Deficiency Anemia

By Victor R. Gordeuk, Gary M. Brittenham, Christine E. McLaren, Margaret A. Hughes, and Louise J. Keating

To determine if elemental carbonyl iron powder is safe and effective therapy for iron deficiency anemia, 20 nonanemic and 32 anemic volunteers were studied. Single doses of 1,000 to 10,000 mg of carbonyl iron (15 to 150 times the 65 mg of iron in the usual dose of ferrous sulfate) were tolerated by nonanemic volunteers with no evidence of toxicity and only minor gastrointestinal side effects. Anemic volunteers (menstruating women who had previously donated blood) were treated with several regimens providing 1,000 to 3,000 mg of carbonyl iron daily in one to three doses for 8 to 28 days. After 12 weeks anemia was corrected in 29 of 32 patients, and serum ferritin was greater than 12 μg/L in 14. Hemoglobin regeneration proceeded at a rate similar to that described for therapy with oral iron salts and parenteral iron dextran. There was no evidence of hematologic, hepatic, or renal toxicity, but mild gastrointestinal side effects occurred in a majority of anemic volunteers. Carbonyl iron is an effective, inexpensive treatment for iron deficiency anemia, is accompanied by tolerable side effects and may have an advantage over therapy with iron salts by substantially reducing or eliminating the risk of iron poisoning in children.

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IRON DEFICIENCY is the most common cause of anemia in the world.[1-3] For the past half century the standard therapy for iron deficiency anemia has been ferrous salts.[4] Recently, however, Crosby suggested that carbonyl iron may be "an ideal therapeutic agent, effective yet lacking in toxicity."[5] Ionic (Fe^{2+}) iron salts such as ferrous sulfate are effective in the correction of iron deficiency anemia, but their use has disadvantages, including the risk of accidental iron poisoning in small children, the occurrence of side effects, and the prolonged duration of treatment required to correct anemia and replenish iron stores. Tablets containing ferrous iron are the second most common cause (after aspirin) of accidental poisoning among small children, leading to as many as 500 hospitalizations and several deaths per year.[5] Side effects with a standard dose of ferrous sulfate occur in 10% to 15% of patients when compared to placebo[6] and result in difficulty in maintaining motivation of patients to comply with therapy. Restoration of hemoglobin to normal with ferrous sulfate requires 3 to 6 months of treatment; replenishment of body iron stores requires therapy for an additional 2 to 4 months.[6-8]

Elemental uncharged iron powder was used early in this century for treatment of iron deficiency anemia and was nontoxic when compared to ferrous salts.[9] However, this elemental iron preparation, which was produced by a hydrogen-reduction process, had poor bioavailability when compared to ferrous salts, possibly due to the relatively large size of the iron particles of about 50 μm[5] and relatively limited reactive surface area.[10] Since then the elemental iron preparation known as carbonyl iron powder has been introduced.[11] "Carbonyl" does not refer to the composition of the iron particles but rather to the manufacturing process in which the controlled heating of vaporized iron pentacarbonyl leads to the deposition of uncharged, elemental iron as submicroscopic crystals that form microscopic spheres of less than 5 μm in diameter The preparation is more than 98% pure.[12] As a food additive, carbonyl iron has been shown to be well absorbed and utilized for hemoglobin synthesis, both in experimental animals and in humans.[12-16] In Sweden, where about half of the fortification iron now used is carbonyl iron, the high bioavailability of this form of iron has been considered one factor contributing to a reduction in iron deficiency.[17] Recent studies in rats have clarified the mechanism of absorption of the finely particulate carbonyl iron now available (Huebers et al, unpublished observations). The conversion of particulate carbonyl iron to soluble ionized iron by stomach acid was a prerequisite for absorption and was limited by the rate of gastric acid production. Subsequent uptake by the intestinal mucosa and absorption were similar with equivalent doses of ferrous iron, although absorption of carbonyl iron occurred over a longer interval. In these studies, the bioavailability of carbonyl iron and ferrous iron was similar with doses of 0.2 to 80 mg Fe/kg body weight.

While not previously used pharmacologically, several studies suggest that as a therapeutic agent, carbonyl iron powder may effectively correct iron deficiency anemia yet be considerably less toxic than iron salts. In rats with iron deficiency anemia, carbonyl iron has been used successfully to correct anemia and replace iron stores.[12,18] In humans, as in dogs, rabbits, and guinea pigs, the estimated lethal dose of oral ferrous sulfate is about 200 mg Fe/kg body weight.[19-21] Human volunteers have taken oral doses of 10,000 mg of carbonyl iron (about 140 mg Fe/kg) "without deleterious effect."[5] Formal toxicity studies of carbonyl iron in rats and guinea pigs found that the LD_0 (the dose that all animals survive) was 10,000 to 15,000 mg Fe/kg and the lethal dose (LD_{100}) was 50,000 to 60,000 mg Fe/kg.[11] These differences in toxicity may be related to differences in the requirements for absorption of iron in the carbonyl and ferrous forms. Fatal amounts of iron may be absorbed through an anatomically intact intestinal mucosa.[20] With ferrous sulfate, all the iron in a soluble ionized form is potentially available for absorption. With carbonyl iron, however, only that fraction solubilized by gastric acid is available for absorption; in addition, the rate of solubilization is restricted by the rate of gastric acid production (Huebers et al, unpublished observations).

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To determine if carbonyl iron is safe and effective for the treatment of iron deficiency anemia in humans, we carried out studies of dosage, toxicity, and side effects for carbonyl iron in 20 nonanemic volunteers and in 32 patients with iron deficiency anemia.

MATERIALS AND METHODS

The use of carbonyl iron was approved by the Committee on Human Investigation at Cleveland Metropolitan General Hospital Studies were conducted under the provisions of a claimed investigational exemption for a new drug (IND No. 22389) of the Food and Drug Administration. All subjects participating in the study were advised of the procedures and attendant risks in accordance with our institution's guidelines and gave informed consent.

Healthy Volunteers

Twenty healthy adult nonanemic volunteers were given orally a reference solution containing 498 mg of ferrous sulfate (100 mg Fe^{2+}) and 100 mg of ascorbic acid in .001 N hydrochloric acid, and at approximately 1-week intervals thereafter various doses of carbonyl iron powder in gelatin capsules were administered orally The doses of iron were given after an overnight fast, and the fast was continued for three to four hours after iron ingestion Ascorbic acid was given along with the ferrous sulfate to permit comparison of our results with previous studies examining the relationship between increase in serum iron concentration and the total amount of iron absorbed [22] Blood samples were drawn initially and at intervals from ½ hour to 24 hours after each dose of ferrous sulfate or carbonyl iron to monitor serum iron, total iron binding capacity (TIBC), percent saturation of TIBC and serum ferritin and to assess liver function as measured by bilirubin, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and alkaline phosphatase, and renal function as measured by serum creatinine. Five volunteers received carbonyl iron in single incremental doses of 100 mg, 1,000 mg, and 5,000 mg at approximately 1-week intervals, four of these volunteers also received a 10,000 mg dose of carbonyl iron. Blood samples were drawn at 0, ½, 1, 2, 3, 4, 5, and 6 hours following the doses of iron Nineteen volunteers received a 3,000-mg dose of carbonyl iron at approximately 1 week or more after the reference dose of ferrous sulfate, and blood samples were drawn at 0, 3, 6, 12, and 24 hours following the doses of iron. Side effects were monitored on a standardized form that included space to record constipation, diarrhea, heartburn, nausea, abdominal cramps, headache, weakness, and "unpleasant taste." The increase in serum iron was used as an indicator of iron absorption

Volunteers With Iron Deficiency Anemia

Thirty-two volunteers were recruited from blood donors to the Northern Ohio Red Cross who met the following criteria: (1) menstruating nonpregnant women between the ages of 18 and 40 years and (2) deferral for repeat blood donation because of hematocrit <38% They received one of several short-term courses of carbonyl iron powder (shown later in Table 2) Gelatin capsules containing either 500 or 1,000 mg of carbonyl iron were used Blood samples were drawn at 0, 1, 3, 6, 9, and 12 weeks for determination of free erythrocyte protoporphyrin (FEP), serum ferritin, serum iron, TIBC, percent saturation of TIBC, and complete blood count (CBC) including hemoglobin, mean cellular volume (MCV), white blood cells (WBC), and platelets. In addition, serum bilirubin, SGOT, SGPT, alkaline phosphatase, and creatinine were measured at 0, 1, and 3 weeks. Side effects were recorded on standard forms as described above at weeks 1 and 3 of the study

Estimation of iron absorption. An estimate for the absorption of carbonyl iron was made by calculating the increase in hemoglobin iron and storage iron between weeks 0 and 12 of the study and using the following equation

Total amount of iron absorbed = increase in hemoglobin iron + increase in storage iron

The increase in hemoglobin iron was calculated using the following equation

Increase in hemoglobin iron = increase in hemoglobin (g/100 mL) × 3.47 mg Fe/g hemoglobin × assumed body weight of 60 kg × 60 mL blood/kg body weight

The increase in storage iron was calculated by assuming that 1 μg/L of serum ferritin represents approximately 10 mg of storage iron if the serum ferritin is greater than 12 μg/L[23] and that storage iron is absent if the serum ferritin is less than 12 μg/L.[24,25] Since at week 0 the serum ferritin was less than 12 μg/L in all but one of our patients, the equation was the following.

$$\text{Increase in storage iron (mg)} = [\text{serum ferritin } (\mu g/L) \text{ at week } 12] - 12\,\mu g/L] \times 10$$

In these calculations neither menstrual loss nor dietary absorption of iron was taken into consideration.

Laboratory Methods

Serum iron, TIBC, and percent saturation of TIBC were determined by the methods of the International Committee for Standardization in Hematology (ICSH).[26] Serum ferritin was analyzed using FER-IRON immunoradiometric assay kits (Ramco Laboratories, Inc, Houston). FEP was measured with a ZnP model 4000 Hematofluorometer (Environmental Sciences Associates, Inc., Bedford, Mass). CBC was determined with a Coulter Counter Model Z_F or a Coulter Counter Model S-Plus II (Coulter Electronics, Inc, Hialeah, Fla). Serum bilirubin, SGOT, SGPT, alkaline phosphatase, and creatinine were measured by an automated method using the Parallel Analytical System (American Monitor Corp, Indianapolis).

Statistical Methods

Data were analyzed using BMDP computer programs.[27]

Studies in nonanemic volunteers. For each of five volunteers given incremental doses of carbonyl iron (Fig 1), the mean change in serum iron achieved over three to six hours after each dose of carbonyl iron was examined using a one-way repeated measures analysis of variance. To test the hypotheses that changes in serum iron increased with increasing doses of carbonyl iron, one-sided paired *t* tests were applied [28] To assure detection of an overall significance level of 05, Bonferroni significance levels were used for individual tests.[29] The mean changes in serum iron over 24 hours after ingestion of 100 mg of ferrous sulfate and 3,000 mg of carbonyl iron in 19 normal volunteers (Fig 2) were compared using repeated measures analysis of variance followed by two-sided paired *t* tests. The binomial test[30] was used to compare the number of overall gastrointestinal side effects experienced by 18 volunteers following a single dose of ferrous sulfate and carbonyl iron. It was also used to compare the numbers of volunteers experiencing individual side effects after each dose of iron.

Studies in anemic volunteers Paired *t* tests were used to compare the mean values for hemoglobin, MCV, FEP, serum ferritin, serum iron, TIBC, and percent saturation of TIBC before and 12

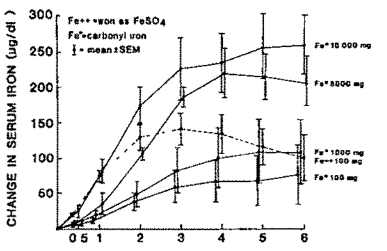

Fig 1. Change in serum iron over six hours following a reference dose of ferrous sulfate and incremental doses of carbonyl iron. N = 5 for all curves except for 10,000-mg dose of carbonyl iron, N = 4. Statistical significance: 1,000 mg and 100 mg, $P = .011$; 5,000 mg and 100 mg, $P = .0004$; 10,000 mg and 100 mg, $P = .0003$; 5,000 mg and 1,000 mg, $P = .0004$; 10,000 mg and 1,000 mg, $P = .0001$.

weeks after carbonyl iron treatment for 32 anemic volunteers (Table 1) Separate one-way analysis of variance procedures followed by pairwise independent sample *t* tests were used to compare the results of the six treatment regimens shown in Table 2 The Bonferroni multiple comparisons procedure was used to assure detection of an overall significance level of 05. The same methods were used to compare the rates of hemoglobin regeneration for volunteers with three levels of initial hemoglobin concentration Pearson's *r* and regression analysis were used to examine the linear relationship between percentage absorption and log cumulative dose of carbonyl iron.[31]

RESULTS

Healthy Volunteers

Figure 1 shows mean response in serum iron over six hours for incremental doses of carbonyl iron alone and a reference dose of 100 mg of iron as ferrous sulfate plus 100 mg ascorbic acid in five healthy volunteers. It should be noted that addition of ascorbic acid to the dose of ferrous sulfate would be expected to enhance iron absorption and to favor ferrous sulfate as far as the magnitude of the increase in serum iron is concerned. The mean (±SEM) maximal increase (141 ± 25 μg/dL) after the reference dose of ferrous sulfate occurred at three hours. This increment was slightly higher than the mean increase of 110 μg/dL reported by Ekenved et al,[22] which corresponded to a whole body absorption of 8.2 mg iron as determined by whole body counting. With an equivalent dose of 100 mg of carbonyl iron, the rise in serum iron was less abrupt but more sustained, with the maximal increase occurring at the end of the six-hour period of observation The rise in serum iron following a dose of carbonyl iron increased progressively with incremental doses of 100, 1,000, 5,000, and 10,000 mg of carbonyl iron. However, in no case was the iron-binding capacity of the serum exceeded. Because of the differences in the pattern of absorption with carbonyl iron and with the reference dose of ferrous sulfate, it was not possible to estimate the whole body absorption of the various doses of carbonyl iron from the serum iron studies or to compare the absorption of the two forms of iron directly.

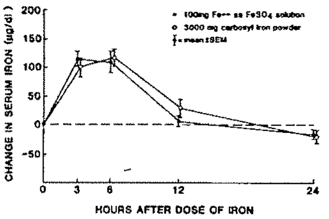

Fig 2. Change in serum iron over 24 hours following 100 mg of iron as ferrous sulfate and 3,000 mg of carbonyl iron; N = 19.

Table 1. Short-Term Carbonyl Iron Therapy for Iron Deficiency Anemia

	Normal	Week 0	Week 12	*P*
Hemoglobin (g/dL)	12 0–16 0	10 8 ± 2	12.9 ± 1	0001
MCV (fL)	81 0–99 0	80 2 ± 1 2	88 3 ± 6	.0001
FEP (μg/dL whole blood)	10–35	48 ± 3	27 ± 1	0001
Serum ferritin (μg/L)	12–250	5.0 ± 5	15 7 ± 1 8	0001
Serum iron (μg/dL)	60–200	71 ± 10	82 ± 6	3652
TIBC (μg/dL)	250–435	417 ± 10	356 ± 9	0001
% Saturation	16–50	17 ± 2	23 ± 2	0559

N = 32 Values shown are mean ± SEM.

Mean responses in serum iron over 24 hours in 19 healthy volunteers after a reference dose of 100 mg of iron as ferrous sulfate plus 100 mg ascorbic acid and after 3,000 mg of carbonyl iron alone are shown in Fig 2. Mean maximal increases in serum iron were nearly the same following both types of iron. The mean (±SEM) maximal increase (115 ± 13 μg/dL) after ferrous sulfate occurred at three hours, while the mean maximal increase (116 ± 16 μg/dL) after carbonyl iron occurred at six hours. No samples were obtained over the next six-hour period, but no statistically significant differences between the two iron preparations were found ($P > .16$) either during the first six hours or during the last 12 hours of observation.

Side effects, including gastrointestinal symptoms and an unpleasant taste sensation, were reported by most volunteers both after 100 mg of Fe^{2+} as ferrous sulfate and after 3,000 mg of carbonyl iron, as depicted in Table 3; these were considered mild by the individuals. Side effect information after carbonyl iron was not available for one of the volunteers. With carbonyl iron the unpleasant taste sensation seemed to be due to eructation. Although overall gastrointestinal ($P = .172$) and other side effects were not statistically different between the two iron preparations, a significantly greater incidence of diarrhea and of abdominal cramping was seen with carbonyl iron. There was no evidence of hepatic or renal toxicity as measured by individual and mean values for serum bilirubin, SGOT, SGPT, alkaline phosphatase, and creatinine drawn initially and at 24 hours.

Volunteers With Iron Deficiency Anemia

Figure 3 and Table 1 depict response to therapy in all 32 anemic volunteers monitored for a 12-week period after starting a course of carbonyl iron treatment lasting from 7 to 28 days and ranging from 1,000 to 3,000 mg per day in single or divided doses. Mean hemoglobin concentration rose to within normal values by the third week and continued to rise throughout the observation period. Mean MCV and FEP returned to normal. All of these changes were statistically

Table 2. Treatment Regimens Used for Iron Deficiency Anemia

Group	Regimen	N	Cumulative Dose of Carbonyl Iron (mg)	Hemoglobin (g/dL)		Ferritin (µg/L)		Estimated Amount of Iron Absorbed (mg)			Amount of Iron Absorbed per Day (mg)	Estimated Absorption of Carbonyl Iron (%)
				Week 0	Week 12	Week 0	Week 12	Hemoglobin	Storage	Total		
I	500-1,000 mg bid to tid for 1-1½ weeks	3	16,000-21,000	10.7 ± .7	13 5 ± .2	3.1 ± 3	14 2 ± 6.1	346 ± 59	47 ± 47	393 ± 48	47 ± 9	2.2 ± 2
II	500 mg tid between meals for 2 weeks	6	21,000	10.1 ± 5	12 7 ± .3	2.8 ± 1	13 7 ± 4.1	336 ± 61	47 ± 30	382 ± 64	27 ± 5	1 8 ± .3
III	1,000 mg bid between meals for 2 weeks	4	28,000	11 1 ± 3	13 0 ± .3	6 6 ± .3	12 8 ± 3.1	235 ± 71	25 ± 25	260 ± 67	19 ± 5	.9 ± .2
IV	1,000 mg daily between meals for 3 weeks	5	21,000	11 5 ± 1	12.9 ± 2	4 8 ± .8	15 7 ± 3 3	183 ± 56	48 ± 27	231 ± 64	12 ± 3	1 1 ± 3
V	500 mg tid with meals for 3 weeks	8	31,500	10.5 ± .4	12.7 ± .4	4.7 ± 1 0	8.5 ± 2 0	282 ± 36	11 ± 11	293 ± 34	14 ± 2	1.9 ± .1
VI	1,000 mg tid with meals for 4 weeks	6	84,000	11.3 ± 2	13 1 ± 2	7.7 ± 1 7	29.8 ± 3 3	232 ± 31	179 ± 33	410 ± 57	15 ± 2	5 ± 1

Values shown are mean ± SEM.

Statistical comparison of treatment regimens at week 12

Hemoglobin overall $P = .783$

Ferritin: overall $P = .006$, V and VI, $P = .0015$; other comparisons, $P >$ 05

Total iron absorbed: overall $P =$ 185

Iron absorbed per day: overall $P =$ 002, I and IV, $P = .003$, I and V, $P = .0195$; I and VI, $P = .041$; II and IV, $P = .003$, other comparisons, $P >$ 05

Carbonyl iron absorption. overall $P =$ 001, I and VI, $P =$ 001, II and VI, $P = .001$; other comparisons, $P >$ 05

Table 3. Side Effects

	In Healthy Volunteers Following a Single Dose of Iron					In Anemic Patients During 1 to 4 Weeks of Treatment With Carbonyl Iron (n = 32)	
	100 mg Fe^{2+} as FeSO$_4$ (n = 20)		3,000 mg Carbonyl Iron (n = 18)				
	No. of Volunteers	(%)	No of Volunteers	(%)	*P*	No of Patients	(%)
Constipation	1	(5)	0	(0)	>.05	9	(28)
Diarrhea	0	(0)	8	(44)	.004	24	(75)
Heartburn	2	(10)	2	(11)	>.05	4	(13)
Nausea	4	(20)	7	(39)	>.05	10	(31)
Epigastric discomfort	1	(5)	3	(17)	> 05	6	(19)
Abdominal cramps	0	(0)	7	(39)	.008	16	(50)
Unpleasant taste	13	(65)	10	(56)	>.05	23	(72)
Headache	6	(30)	6	(33)	>.05	10	(31)
Weakness	2	(10)	2	(11)	>.05	5	(16)

significant. Mean serum iron and mean percent saturation of the TIBC did not change significantly during the study. Mean ± SEM serum ferritin concentration, which was 5.0 ± 5 µg/L initially, rose during carbonyl iron therapy to a peak value of 40.0 ± 4 6 µg/L at week 1 and then declined to a final value of 15.7 ± 1.8 µg/L at week 12, which was significantly greater than the initial concentration. At the end of the study period, hemoglobin was normal (greater than 12 0 g/dL) in 29 of the 32 volunteers, and serum ferritin was greater than 12 µg/L in 14 of the 32 volunteers. Mean serum ferritin in these 14 patients was 26 ± 2 µg/L. Of the 14 patients with normal ferritin at the end of the study, 12 had initial hemoglobin of >11.0 g/dL.

The responses in mean hemoglobin and serum ferritin and estimates of the mean amounts of iron absorbed for each of six different regimens of short-term carbonyl iron therapy used in the anemic volunteers is shown in Table 2. Although the final hemoglobin concentrations were similar among the groups, there were significant differences in the final serum ferritin concentrations. In group VI, which received the largest dose of iron for the longest period of time, serum ferritin was significantly higher than in group V. In group VI hemoglobin was normal and serum ferritin was >12 µg/L in all volunteers after 4 weeks of carbonyl iron therapy.

Hemoglobin regeneration. The response to carbonyl iron therapy as measured by daily increase in hemoglobin concentration was calculated for each patient. The rate of rise in hemoglobin varied according to the initial hemoglobin concentration but not the treatment protocol. (1) With an initial hemoglobin of 8.9 to 10.0 g/dL, the mean daily increment (±SEM) in hemoglobin was 0.11 ± .01 g/dL during the first 3 weeks of the study when carbonyl iron was taken for all or part of this time. (2) With an initial hemoglobin of 10.0 to 11.0 g/dL, the mean daily response in the first 3 weeks was 0.09 ± .01 g/dL, and (3) with an initial hemoglobin of 11.0 to 12.0 g/dL, it was .04 ± .01 g/dL. The hemoglobin regeneration rate for case 3 was significantly lower than for cases 1 (*P* = .0007) and 2 (*P* = .0004) As mentioned previously, the mean hemoglobin concentration continued to rise throughout the study period, even following the completion of carbonyl iron therapy. During weeks 6 to 12, when carbonyl iron therapy had been completed for at least 2 weeks, there was a mean daily increase in hemoglobin of .01 ± .01 g/dL; this increment was seen for all three categories of initial hemoglobin concentration.

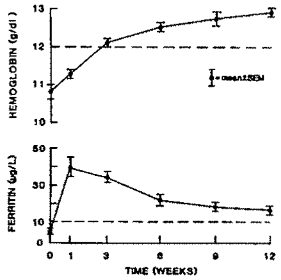

Fig 3. Change in hemoglobin and serum ferritin concentrations with short-term carbonyl iron therapy in anemic volunteers; N = 32.

Absorption of carbonyl iron. An estimate of the total and daily amount of carbonyl iron absorbed during the study was made for each patient, as shown in Table 2. Estimated total amounts of iron absorbed for the various treatment groups ranged from mean (±SEM) values of 232 ± 64 to 410 ± 57 mg, but there were no statistically significant differences among the six groups. However, when expressed as the amounts of iron absorbed per day, the range was 12 ± 3 to 47 ± 9 mg, and there were significant differences among the groups. Group I, with the shortest duration of therapy, had a greater estimated absorption per day than groups IV, V, and VI, and group II had a greater estimated absorption than group IV There were also significant differences in estimated percentage absorption of the cumulative dose of carbonyl iron among the groups. Group I and II, which received the smaller cumulative doses, had a greater estimated percent absorption than group VI, which received the largest cumulative dose. Analysis of the estimates of percent absorption for all 32 volunteers revealed a significant negative correlation with the log of the cumulative dose of carbonyl iron ($r = -.62$; $P < .001$).

Side effects and toxicity. Side effects, shown in Table 3, were recorded at 1 and 3 weeks of the study in the 32 anemic patients. Gastrointestinal symptoms and an unpleasant taste were reported by a majority of patients. The side effects were considered by the individuals to be mild in most instances, and only three patients considered them severe enough to warrant discontinuing therapy. In 607 patient-days of therapy with carbonyl iron, there was no evidence for hematologic, hepatic, or renal toxicity as judged by individual and mean values for WBC, platelets, bilirubin, SGOT, SGPT, alkaline phosphatase, and creatinine measured at 0, 1, and 3 weeks.

Cost. Carbonyl iron powder was purchased from GAF Corp (Linden, NJ) at a price of 2 cents per gram. For comparison, the current average price of ferrous sulfate (20% iron) is 35 cents per gram iron, based on manufacturers' listings in the 1985 *Drug Topics Red Book* for the purchase of 100 capsules or tablets The cost of the treatment regimens with carbonyl iron in this study ranged from $ 37 to $1.95; standard-dose ferrous sulfate therapy for 4 weeks would have cost, on the average, $1.78 In this comparison, we were unable to take into consideration any additional cost that would result were carbonyl iron to be marketed as a pharmaceutical

DISCUSSION

Our results suggest that carbonyl iron is safe and effective for the treatment of iron deficiency anemia, is associated with tolerable side effects, and may have a cost comparable to that of ferrous sulfate. We investigated its use as a pharmacologic agent because of studies indicating that carbonyl iron has very low toxicity when compared to standard iron therapy with ferrous salts.[5,11,18] In preliminary dosage and toxicity studies, healthy volunteers were given single doses of carbonyl iron ranging from 100 to 10,000 mg with no evidence for toxicity and with mild side effects. It is of note that the 10,000-mg dose of carbonyl iron given to four volunteers is over half the lethal dose of iron as ferrous sulfate (about 14,000 mg in a 70-kg male).[19] In five healthy volunteers (Fig 1), the increase in serum iron following a dose of carbonyl iron was less abrupt but more sustained than that from an equivalent dose of ferrous sulfate. Similar results have been obtained in studies of the pattern of absorption of the two forms of iron in rats (Huebers et al, unpublished observations), but no direct studies of the absorption of carbonyl iron in humans are available. Because of the difference in the pattern of absorption between carbonyl iron and ferrous sulfate, relative amounts of iron absorbed with the two iron preparations could not be determined from the data shown in Figs 1 and 2.

A short course of 1 to 4 weeks of carbonyl iron was used to treat patients with mild and moderate iron deficiency anemia. The daily doses of carbonyl iron (1,000 to 3,000 mg in capsules, each containing 500 to 1,000 mg) were 5 to 15 times the usual recommended dose of iron as ferrous sulfate (200 mg in tablets, each containing 65 mg),[32] yet side effects were tolerable and there was no evidence of hematologic, hepatic, or renal toxicity. In rats and guinea pigs, the acute lethal dose of carbonyl iron powder lies between an LD_0 of 10,000 mg/kg and an LD_{100} of 60,000 mg/kg.[11] If we extrapolate these data to humans, a small child of 10 kg body weight ingesting the largest amount of carbonyl iron prescribed in this study, 84 1-g capsules over 28 days, would have ingested an amount well below the LD_0. For comparison, the estimated lethal dose of ferrous iron (200 mg Fe/kg)[19] for such a child would be 37 ferrous sulfate tablets (65 mg Fe each), or a 10-day supply for standard adult therapy.

The increases in hemoglobin concentration with carbonyl iron therapy in this study are comparable to those described in previously published studies reporting response to ferrous sulfate[7,33-36] and to parenteral iron.[8,37] It has been shown that the rate of rise in hemoglobin in response to parenteral iron therapy varies inversely with the initial hemoglobin concentration.[37] Our results with carbonyl iron also show an inverse relation between the rate of hemoglobin regeneration and the initial hemoglobin concentration similar to that reported in the study with parenteral iron.[37] The continued slight mean rise in hemoglobin of .01 g/dL per day following discontinuation of carbonyl iron therapy may have occurred in response to iron that had been absorbed and stored during the period of carbonyl iron therapy, or it may have been due to dietary iron; similar results have been observed both with patients on prolonged ferrous sulfate therapy and with untreated patients responding to dietary iron.[36]

In the 14 patients who achieved serum ferritin >12 µg/L at the end of 12 weeks of observation, mean serum ferritin was 26 µg/L. This concentration is almost identical to the mean value of 25 µg/L observed in a population of women who were repeat blood donors,[38] suggesting that in these patients some iron stores may have been replaced. Further examination of these results shows that while serum ferritin was >12 µg/L in 12 of 19 (63%) patients whose initial hemoglobin was greater than 11.0 g/L, it was above this concentration in only 2 of 13 (15%) patients whose initial hemoglobin was less than 11.0 g/dL. This suggests that although in most patients with mild iron deficiency anemia (hemoglobin 11.0 to 12.0 g/dL) enough iron was absorbed during short-term carbonyl iron therapy to correct anemia and to replace some storage iron, in most patients with moderate anemia (hemoglobin 8.9 to 11.0 g/dL) all of the iron absorbed was ultimately used in erythropoiesis. However, in group VI, which received the greatest amount of carbonyl iron, the final hemoglobin concentration was in the normal range, and the serum ferritin concentration was >12 µg/L in all subjects, suggesting that even a short-term course of carbonyl iron can correct anemia and may at least partially rebuild iron stores in patients with mild iron deficiency anemia. It can be noted parenthetically that in our study, as has been previously reported,[39-41] there was a rise in serum ferritin during iron therapy, which apparently did not reflect storage iron (Fig 3). It was for this reason that we used the final serum ferritin values, obtained 56 to 77 days after completion of iron therapy, as the indicator of storage iron. It should also be pointed out that in this study we have not shown that the final hemoglobin concentration, though in the normal range, was the optimal level for the individual patients. It is possible that the hemoglobin concentration continued to rise after 12 weeks (Fig 3) and that some storage iron was used for hemoglobin production after 12 weeks. The 12-week serum ferritin cannot, therefore, be viewed as a definitive measure of storage iron achieved by carbonyl iron therapy in this study

Side effects occurred in most anemic patients given carbonyl iron in this study, predominantly diarrhea, abdominal cramps, and an unpleasant taste sensation. Although such side effects are a potentially serious drawback to the use of carbonyl iron therapeutically, it should be emphasized that most of the individuals considered them mild and tolerable. In the nonanemic volunteers who received a single dose of ferrous sulfate and a single dose of carbonyl iron that contained a 30-fold greater amount of elemental iron, there were no significant differences in overall gastrointestinal side effects between the preparations, but when abdominal cramping and diarrhea were considered separately, their incidences were significantly more frequent with carbonyl iron. Also, since giving a placebo for iron deficiency anemia is associated with a significant incidence of side effects,[42] defining the true incidence of side effects related to carbonyl iron requires a randomized double-blind trial comparing carbonyl iron, ferrous sulfate, and a placebo. Such a trial is now in progress.

Although the exact mechanism of intestinal absorption of iron is still not known, we believe the evidence from this study suggests that absorption of the doses of carbonyl iron used here proceeds in a controlled physiologic manner. Supporting observations include the following: (1) In healthy volunteers, incremental doses of carbonyl iron led to increasing increments in serum iron following the dose (Fig 1); (2) the iron-binding capacity of the serum was not exceeded even with large doses of up to 10,000 mg of carbonyl iron (Fig 1); (3) following an increase in the serum iron after a dose of carbonyl iron, there was a decline in the serum iron at 12 and 24 hours as seen with ferrous sulfate (Fig 2); and (4) in the anemic patients, intact mucosal selectivity in the absorption of carbonyl iron was suggesting by the declining percent absorption associated with increasing cumulative dose of iron and the length of treatment, as is seen with ferrous sulfate therapy.[34] Although an original observation by Volkheimer[43] described nonphysiologic "persorption" of carbonyl iron particles directly into the portal blood in dogs, the doses he gave (when adjusted for body weight) were approximately 80 times the largest dose given to healthy volunteers and 700 times the largest dose given to anemic patients in our study. Previous studies in our laboratory[44] showed "no evidence of particulate or elemental iron" on histologic and electron microscopic examination of the livers of rats fed with extremely large quantities of carbonyl iron (2.5% wt/wt of the diet) for up to 6 months. Studies in rats using radioactive carbonyl iron have also found no evidence for "persorption" (Heubers et al, unpublished observations).

In this study brief courses of 1 to 4 weeks of carbonyl iron therapy were examined to determine the extent to which anemia could be corrected and storage iron replenished with such short-term therapy. Since an earlier study with animals had shown that a single large oral dose of carbonyl iron corrected iron deficiency anemia and replaced storage iron,[19] we wished to find the largest safe daily therapeutic dose of carbonyl iron with side effects no greater than those associated with ferrous sulfate. In addition, with the 12-week observation period, the short-term therapy allowed us to estimate the amount of iron absorbed as described in Materials and Methods. The mean (±SEM) amount of carbonyl iron absorbed per day for the various treatment groups was estimated to be 12 ± 3 to 47 ± 9 mg (Table 3). These amounts compare favorably with the amount of orally administered iron that the marrow can use for erythropoiesis in mild and moderate iron deficiency anemia, approximately 10 to 20 mg per day,[45,46] and coincide with the finding that in some of our patients, iron in excess of what was neeeded for erythropoiesis was absorbed and may have been used to replenish storage iron.

In summary, our results provide evidence that carbonyl iron is a safe, effective, well-tolerated, and inexpensive therapy for iron deficiency anemia. Most important, carbonyl iron may have an advantage when compared to the ferrous salts now in use by decreasing accidental iron poisoning in children. Although there is no direct experience with use of carbonyl iron in children, experience in normal volunteers[5] and in animals[11,18] suggests that the risk of toxicity and poisoning in children would be greatly reduced when compared to ferrous salts. Patients with mild anemia may correct anemia and rebuild iron stores with even a short course of carbonyl iron, but the optimal dose and duration of therapy for more severe anemia remain to be determined.

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High Frequency of *N-ras* Activation in Acute Myelogenous Leukemia

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Using the NIH/3T3 cell transfection assay, activated cellular oncogenes have been detected in around 10% to 20% of human tumors. From a series of DNA preparations from tissues infiltrated with acute myelogenous leukemia (AML), 50% (3/6) caused transformation of NIH/3T3 cells. Thus AML appears to be the human tumor with the highest frequency of oncogenes detected by DNA transfection. In each case the oncogene involved was *N-ras*, a member of the *ras* gene family. Biologic and clinical parameters of AML patients with and without *N-ras* oncogenes in their tumors are discussed.

EVIDENCE ARISING FROM several different approaches has implicated a small number of cellular genes or proto-oncogenes as frequent targets for genetic alterations that can lead human cells along the pathway to malignancy. These cellular genes were initially identified as the transduced cellular "*onc*" genes of acute transforming retroviruses.[1,2] More recently, it has been found that some of the very same cellular genes may be activated as oncogenes in human tumors completely independent of retrovirus involvement.[3-5]

One important approach to the detection of human transforming genes has involved DNA transfection. A wide variety of human tumors contain DNA sequences capable of inducing morphologic transformation upon transfection of NIH/3T3 cells, a continuous mouse embryo cell line.[6-11] The vast majority of oncogenes detected in this manner have been found to be related to a small proto-oncogene family, *ras*. Recent studies have also implicated other proto-oncogenes in human malignancies. DNA rearrangements such as translocation and gene amplification involving human proto-oncogenes have been associated with specific forms of cancer.[12-16]

Human acute myelogenous leukemia (AML) is a highly malignant hematologic neoplasm. There have been a few reports of *ras* oncogenes in neoplastic tissues or cultures derived from AML patients, as well as a recent study demonstrating amplification of the proto-oncogene *c-myb* in one AML cell line.[11,17-19] This present study of six patients with AML was undertaken to examine further the frequency of oncogene activation in this disease, as well as to characterize what clinical parameters, if any, might be associated with oncogene activation.

MATERIALS AND METHODS

DNA transfection. Pretreatment marrow aspirates or autopsy materials were selected if they were heavily (>75%) infiltrated with acute nonlymphocytic leukemia cells as judged by a pathologist. Specimens selected for study were frozen promptly to prevent DNA degradation, and tumors were homogenized in liquid nitrogen, lysed in .5% sodium dodecyl sulfate (SDS) containing 10 mmol/L Tris, 2 mmol/L EDTA, and 150 mmol/L NaCl and treated overnight with proteinase K (.2 mg/mL at 37°C). Proteins were extracted with phenol, then with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1), and then with chloroform and isoamyl alcohol (24:1). DNA was precipitated with ethanol, dried, and suspended in 1 mmol/L Tris and .2 mmol/L EDTA.

The DNA transfection assay was performed essentially as described.[20] Briefly, NIH/3T3 cells were plated in 10-cm petri dishes at 1.3×10^5 cells per plate 20 hours before transfection. DNA (30 μg) was suspended in .25 mol/L $CaCl_2$ and precipitated by addition to an equal volume of 50 mmol/L HEPES, 280 mmol/L NaCl, and 1.5 mmol/L NaH_2PO_4 during gentle nitrogen bubbling. After 30 to 60 minutes, precipitates were resuspended and added to the NIH/3T3 cell monolayer. Precipitates were removed 20 hours later. Culture medium was changed twice weekly, and focus formation was scored at 14 to 21 days. Genomic DNA from the T24 human bladder carcinoma cell line was used as a positive control. T24 DNA yielded 5 to 15 focus-forming units per plate.

Southern blot analysis. DNAs (15 μg lane) were electrophoresed at 30 V in 1% agarose and transferred to nitrocellulose by the method of Southern.[21] Molecular probes were nick-translated with $\alpha^{32}P$ dCTP, denatured, and hybridized overnight at 42°C in the presence of 50% formamide, 750 mmol/L NaCl, 75 mmol/L Na citrate 1X Denhardt's solution, 20 mmol/L phosphate buffer, and 100 μg/mL sheared, denatured salmon sperm DNA. The filters were washed three times with 300 mmol/L NaCl, 30 mmol/L Na citrate, and .1% SDS at room temperature and then with 15 mmol/L NaCl, 1.5 mmol/L Na citrate, and .1% SDS twice at 50°C. After air drying, the filters were exposed two to six hours at −80°C on an intensifying screen and developed in a Kodak RP X-O-Mat processor. DNA probes of human origin included *N-ras*, *K-ras*, *H-ras*, *c-myc*, *c-myb*, and *N-myc*.

Immunoprecipitation of the ras *gene product.* Two days following plating a density of 3×10^6 cell cultures were methionine-starved for 30 minutes and labeled with .3 mCi L-^{35}S-methionine/mL for three hours. After washing with phosphate-buffered saline (PBS), the cells were lysed in 1% Triton-X-100, .1% SDS, .5% sodium deoxycholate, .1 mol/L NaCl, 10 mmol/L phosphate buffer (pH 7.4) containing 1.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 100 kallikrein inactivator units of aprotinine/mL. After centrifugation at 30,000 g for 15 minutes, 2×10^7 trichloroacetic-acid precipitable counts of the cleared lysates were incubated with anti-*ras* monoclonal antibody Y13-259[22] at 4°C for 45 minutes. Bound p21 was precipitated with protein A sepharose coated with goat antirat IgG, washed three times, and boiled in sample buffer containing 2-mercaptoethanol. Samples were electrophoresed at 250 V for three hours along with ^{14}C-labeled protein standards on a 12.5% bis-acrylamide slab gel. Following fixation, fluorography with Enhance, and rehydration, the slab gel was dried and exposed overnight on an image-intensifying screen at −80°C.

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RESULTS

High-frequency activation of N-ras *oncogenes in primary AML.* Table 1 provides information on six patients with AML included in our study. DNA extracted from tumor tissues was assayed in the NIH/3T3 transfection assay. As shown, affected tissues of three of the patients scored as positive. In each case, the transforming activity persisted in a second cycle of transfection. The great majority of activated oncogenes associated with human malignancies and detected in the NIH/3T3 transfection assay are members of the *ras* gene family. *H-* and *K-ras* were initially detected as the transduced *onc* genes of transforming retroviruses,[23] while *N-ras* was initially detected as the oncogene of a human neuroblastoma cell line.[24] Thus we probed first-cycle transfectants with radiolabeled DNA fragments derived from human *H-*, *K-*, and *N-ras*, respectively. Neither *H-* nor *K-ras* probes detected new, hybridizable DNA fragments (data not shown). However, each of the transfectant DNAs contained new DNA fragments detectable under stringent hybridization conditions with the *N-ras* probe (Fig 1). Similar results were obtained with second-cycle transfectants, indicating the segregation of human *N-ras* sequences with the transformed phenotype of the cells. Differences in the sizes of *Eco*RI-digested *N-ras* fragments imply DNA rearrangements outside the region that is required for *N-ras*-transforming function during the transfection and/or integration process.

Recent studies have documented that activating lesions at codon 12 or 61 in the *ras* coding sequence and the consequent amino acid substitution at either of these positions can alter the electrophoretic mobility of the transforming protein. These alterations induce characteristic retarding or accelerating effects of p21 migration.[25,26] Thus we examined transfectants containing *N-ras* oncogenes derived from the three positive AML patients by immunoprecipitation analysis for their pattern of p21 electrophoretic mobility using broadly cross-reactive anti-p21 monoclonal antibody Y13-259. As shown in Fig 2, representative transfectants of each positive AML exhibited abnormal p21 species; when compared with normal NIH/3T3 cells, the level of p21 expression was consistently elevated in all transfectants analyzed. The increased expression of p21 in AML transfectants is attributed to the presence of the added human *N-ras* oncogene because DNA hybridization experiments established that the transfectants contained only human *N-ras* and not human *H-ras* or *K-ras* oncogenes or amplified copies of any mouse *ras* genes. However, direct identification of human *N-ras* encoded p21 proteins is not possible as yet due to the unavailability of *N-ras*-specific antibodies. Each of the three *N-ras* oncogene products demonstrated a similar migration, suggesting that a similar mutational event might be responsible for the activation of *N-ras* oncogenes in each of these cases.[26]

Table 1. DNA Transfection Analysis of Six Cases of Acute Myelogenous Leukemia

Patient	Age	Sex	Survival (months)	Tissue	Transforming Activity (foci/plate) First Cycle	Transforming Activity (foci/plate) Second Cycle
1	45	F	30+	Marrow	2/8	45/8
				Normal skin	0/16	—
2	13	M	8	Spleen	2/16	7/8
3	70	M	12	Spleen	1/16	66/8
4	62	M	2	Spleen	0/16	—
5	57	F	9	Spleen	0/16	—
6	55	M	12	Spleen	0/16	—

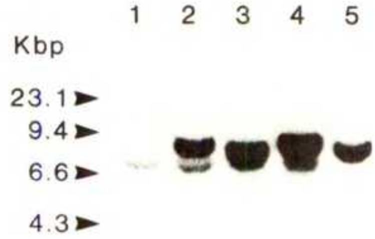

Fig 1. ***N-ras*** **oncogenes in NIH/3T3 transfectants of human AML DNAs. DNA samples of 15 μg each from first-cycle NIH/3T3 transfectants of AML patients 1, 2, and 3 (lanes 2, 3, and 4) were digested with** ***Eco*****RI and run in a 1% agarose gel, blotted to nitrocellulose paper, and probed with the** ***Pvu*****II fragment of the human** ***N-ras*** **gene.[11] NIH/3T3 (lane 1) and human placenta (lane 5) DNAs served as controls.**

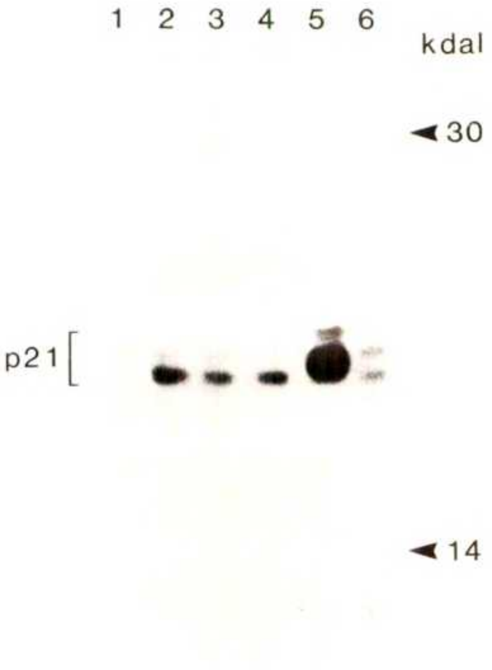

Fig 2. Electrophoretic patterns of ***N-ras*****-oncogene-encoded p21 proteins in NIH/3T3 transfectants of human AML DNAs. Control NIH/3T3 (lane 1) and NIH/3T3 transfectants derived from DNAs of patients 1, 2, and 3 (lanes 2, 3, and 4) were compared with transfectants of T24 bladder carcinoma containing a position 12-mutated** ***H-ras*** **(lane 5) and of SW1271 lung carcinoma containing a position 61-mutated** ***N-ras*** **(lane 6). After immunoprecipitation with monoclonal antibody Y13-259, p21 electrophoretic mobilities were compared by SDS 12.5% polyacrylamide gel electrophoresis.**

Somatic activation of N-ras *in an AML patient.* The family history of patient 1 indicates a high incidence of hematopoietic abnormalities, including paroxysmal nocturnal hemoglobinuria (PNH), neutropenia, acute leukemia, and Hodgkin's disease. It is conceivable that families with site-specific tumor aggregation[27] inherit a propensity for the development of the tumor by vertical passage of an altered or activated oncogene. This prompted us to investigate whether the DNA from this patient's normal tissue might contain an activated cellular oncogene. DNA extracted from apparently normal skin scored repeatedly negative in the NIH/3T3 transfection assay, with a total of 16 plates analyzed under conditions where the activated *N-ras* gene of this patient's leukemia tissue was readily detectable (Table 1). These findings suggest that *N-ras* activation in this patient occurred as a somatic event in agreement with the results of similar studies of other tumors.[28-30]

Hybridization analysis of human AML DNAs for aberrations affecting other human proto-oncogenes. Recent studies have identified rearranged *c-myc* in Burkitt's lymphoma as well as amplification of *c-myc*-related genes in a number of other human malignancies.[12,13,15,18,31] In addition, *c-myb* has been shown to be amplified in one AML cell line.[19] Thus we surveyed DNAs of our series of primary AMLs for evidence of gene rearrangements or gene amplification affecting either of these proto-oncogenes. There was no detectable amplification or gross DNA rearrangement with respect to the indicated restriction enzyme involving either *myb* (Fig 3) or *myc* (data not shown) in any of the samples analyzed, indicating that such alterations are not commonly detectable in AML. Moreover, analysis of *Eco*RI- or *Bam*HI-digested genomic DNA failed to reveal DNA rearrangement or gene amplification of *H-ras*, *K-ras*, *N-ras*, or *N-myc* (data not shown).

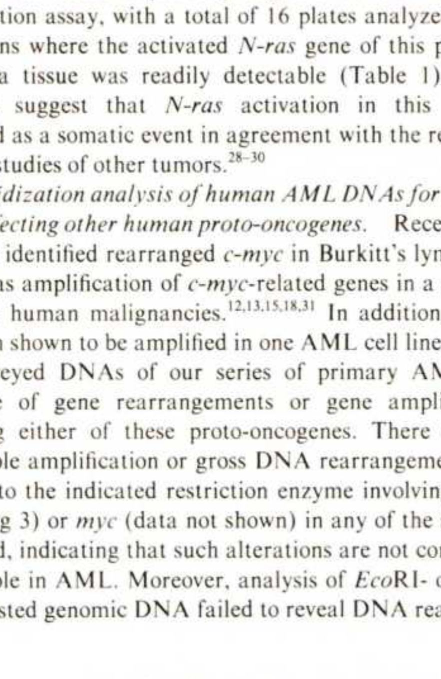

Fig 3. Analysis of human AML DNAs for aberrations affecting other proto-oncogenes. DNA samples (15 μg) from seven AML patients (lanes 1 through 7) and human placenta (lane 8) were digested with *Hind*III and hybridized to the 2.6-kb and 1-kb *Eco*RI fragments of human *c-myb*.[40]

DISCUSSION

Our observations that 3 of 6 tumors contained an activated *N-ras* oncogene suggest that approximately 50% of the neoplastic tissues from patients with AML will score as positive in the NIH/3T3 DNA transfection assay. In considering our cases in concert with the five cases of AML examined in other studies,[11,17,18] a total of 6 of 11 were positive. There have only been a few studies of the frequency of *ras* oncogene activation in a large series of tumors of the same types. In breast and urothelial tumors, the frequency of *ras* oncogenes detected by DNA transfection was <5% and around 7.5%, respectively.[29,30] The combined results of several studies have indicated that lung and colon carcinomas exhibit *ras* oncogenes detected by analogous approaches in around 10% to 30% of primary tumors or tumor cell lines.[10,25] Thus AML appears to exhibit the highest frequency of *ras* oncogene activation of any human tumor so far studied.

It is possible that oncogene activation detectable in the NIH/3T3 transfection assay reflects some biologic property intrinsic to the tumor subtype in question. NIH/3T3 cells lose density-dependent growth inhibition and become tumorigenic in nude mice after transfection with activated *ras* genes. Thus it is conceivable that tumors expressing *ras* oncogenes might be relatively more aggressive with respect to tissue invasiveness and/or metastatic potential. In our present series of AMLs, such clinical parameters as leukocyte count at presentation, degree of organ infiltration, response to therapy, and survival did not seem to be significantly correlated with the presence or absence of detectable *N-ras* oncogenes. However, further studies of *N-ras* activation in AML are needed to validate these findings and explore further whether detection of this marker is associated with any subgroup of patients with distinctive biologic and/or clinical behavior of their disease.

Previous studies have demonstrated that in a few tumors where normal tissues from the same patient were available, *ras* oncogenes were activated by point mutations as somatic events detectable only in the tumor.[28-30] In our present survey of human AMLs, we were able to analyze DNA of normal cells from one patient whose tumor was positive for an *N-ras* oncogene. This patient's family history indicated a high incidence of hematopoietic abnormalities, including PNH, neutropenia, acute leukemia, and Hodgkin's disease. The absence of oncogenes detectable by DNA transfection in normal tissues of this patient suggests the somatic activation of an *N-ras* oncogene in this tumor. While genetic transmission of an *N-ras* oncogene cannot be implicated in the high familial incidence of hematopoietic abnormalities, its selection within the tumor implies that it was likely involved in this patient's tumor development.

The mobilities of *ras* p21 proteins altered by the most common activating lesions at codon 12 or 61 are often altered in ways that readily discriminate them.[26] Our present findings demonstrated that the mobilities of p21s encoded by each of the *N-ras* oncogenes of our AML patients analyzed were similar, but intermediate in mobility compared to those of typical p21s containing position 12 or 61 mutations. These findings indicate that these oncogenes may possess a common mutation, whose effect on p21 mobility doesn't allow ready classification. The precise mechanisms by which these oncogenes are activated awaits their molecular cloning and sequence analysis. In a report published after submission of our study, Bos et al[32] also detected *N-ras* oncogenes at high frequency in AML. Moreover, these investigators presented evidence that point mutation at codon 13, rather than at one of the common sites for activation at position 12 or 61, is responsible for the acquisition of transforming properties by *N-ras* oncogenes in their cases of AMLs. It will be of interest to determine whether position 13 mutations account for any of the activating lesions in our present studies of *N-ras* as agents of AML patients.

Chromosomal aberrations such as translocations, inversions, and deletions occur in the majority of AMLs.[33] Specific translocations have been implicated in the activation of proto-oncogenes in Burkitt's lymphoma[12] and chronic myelogenous leukemia.[14] Although it was not possible to perform cytogenetic analysis of our present cases, the karyologic anomalies observed in AML involve chromosome 1 where the *N-ras* proto-oncogene resides in less than .5% of cases.[34] In fact, in those cases where chromosome 1 anomalies are observed in AML, the break point is more often at the long arm of chromosome 1,[34,35] while the *N-ras* site is on the short arm, at 1p11 to 1p21.[34] Thus the observation of a high frequency of *N-ras* activation in AML coupled with the very rare observation of chromosomal anomalies in the vicinity of this gene strongly suggests that a specific primary karyologic change is not involved in *N-ras* activation.

Myc and *myb* oncogenes of acute transforming retroviruses commonly induce myeloid leukemias.[1,2] *Ras* oncogenes have been shown to induce alterations in the growth and differentiation of cells within the myeloid lineage, in some cases associated with the acquisition of malignant properties.[36,37] In view of these findings as well as reports of amplified *myb*[19] and *myc*[38,39] DNA sequences, respectively, in two different myeloid leukemia cell lines, we investigated seven primary AMLs for evidence of gene amplification or gross DNA rearrangement involving *myc* and related *N-myc*, *myb* and the three *ras* oncogenes. None was detected, implying that such alterations are not commonly associated with this tumor. Continued search for specific aberrations affecting the small set of cellular genes that are known to give rise to oncogenes may help to define other genes that contribute to the pathogenesis of this highly malignant tumor.

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Loss of the Largest von Willebrand Factor Multimers From the Plasma of Patients With Congenital Cardiac Defects

By Joan C. Gill, Allen D. Wilson, Janet Endres-Brooks, and Robert R. Montgomery

We identified a consecutive series of 12 children with noncyanotic congenital cardiac lesions with loss of the largest plasma von Willebrand factor (vWF) multimers determined by SDS-agarose electrophoresis. Seven had previous histories of mucocutaneous hemorrhage; ten had a prolonged bleeding time. Analysis of the factor VIII molecular complex revealed that six patients had reduced vWF measured both immunologically (vW:Ag) and by ristocetin cofactor assay (vW:rist). All had normal or borderline normal factor VIII procoagulant (F VIII) concentrations. Three children had prolonged partial thromboplastin times due to concurrent factor XII deficiency; none had laboratory evidence of intravascular coagulation. Five of the children were restudied after surgical correction of their cardiac lesions. Four had normalization of vWF multimers; the fifth, whose vWF was abnormal postoperatively, had a residual pressure gradient across a previous pulmonary artery banding site. Multimeric abnormalities were not found in the parents of three patients. Thus some patients with noncyanotic congenital heart disease may have an acquired abnormality of vWF that is normalized with correction of the abnormal hemodynamic state.

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THE PLASMA FACTOR VIII molecular complex is composed of two proteins: factor VIII (F VIII), the procoagulant protein that functions as a cofactor in factor IXa activation of factor X in blood clotting; and von Willebrand factor (vWF), the large multimeric protein that supports platelet adhesion.[1,2] Both proteins can be measured immunologically and functionally; thus both qualitative and quantitative abnormalities of vWF are possible and have been described.[1-3] Patients with inherited von Willebrand disease (vWD) of type II have qualitative defects of vWF; these defects are manifested by mild to moderate mucous membrane and cutaneous hemorrhages, prolonged bleeding times, decreased vWF-ristocetin cofactor activity (vW:rist), and absence of the large vWF multimers demonstrated by sodium dodecyl sulfate (SDS)–agarose electrophoresis of plasma.[2,3] Acquired defects of vWF have been reported in patients with disseminated intravascular coagulation (DIC),[4,5] the episodic form of hemolytic uremic syndrome,[6] acute myocardial infarction,[7] and myeloproliferative syndrome.[8]

We recently had the opportunity to study nine patients with ventricular septal defect (VSD), one with atrial septal defect (ASD), and two with aortic stenosis. Each patient was referred for evaluation of hemorrhagic symptoms or a long bleeding time. All 12 patients had apparent acquired absence of the largest vWF multimers by SDS-agarose electrophoresis.

MATERIALS AND METHODS

Patients. The 12 consecutive patients were referred for either evaluation of hemorrhagic symptoms or abnormal preoperative hemostatic screening tests. Laboratory evaluation was performed at Milwaukee Children's Hospital Clinical Laboratory and the Hemostasis Reference Laboratory of the Blood Center of Southeastern Wisconsin, Milwaukee. Careful histories and physical examinations were conducted for all patients.

Collection of blood samples. Plasma was obtained from venous blood sampled by a double-syringe technique and anticoagulated with sodium citrate (3.2%). Platelet-rich plasma was obtained by centrifugation of anticoagulated whole blood at 1,600 g for five minutes and platelet-poor plasma by centrifugation at 4,800 g for 20 minutes in a refrigerated centrifuge.

Standard coagulation assays. Bleeding times were calculated with the Simplate device (General Diagnostics, Morris Plains, NJ) according to the manufacturer's suggestions. Prothrombin (PT) and partial thromboplastin (PTT) times were determined by recalcification assays on an MLA Electra-700 (Dade, Mount Vernon, NY). Platelet counts were performed on an ELT-8DS (Ortho, Boston) and confirmed by visualizaton of Wrights-stained peripheral blood smears by light microscopy. Fibrinogen assays were performed on a fibrometer utilizing Data-Fi fibrinogen reagents (Dade, Miami) and fibrin(ogen) degradation products by the Thrombo-Wellco Test (Burroughs-Wellcome, Research Triangle Park, NC).

Studies of the factor VIII molecular complex. Factor VIII procoagulant activity (F VIII:C) was determined by a one-stage method using F VIII–deficient plasma as a substrate.[9] Quantitative measurement of vWF (vW:Ag) was performed using a rocket immunoelectrophoretic technique in .9% agarose with rabbit antihuman vWF.[10] Ristocetin cofactor activity of vWF (vW:rist) was determined using formalin-fixed human platelets.[11] Multimeric analysis of vWF was determined by electrophoresis of plasma in .65% Seakem HGT-(P) agarose gels (FMC Corp, Rockland, Me) in the presence of .1% SDS and visualized by radiolabeled rabbit antihuman vWF and autoradiography as previously described.[12] The reference standard for factor VIII and vWF assays was pooled plasma from 50 normal donors (stored at −80°C).

RESULTS

During the period from June 1982 to December 1984, nine patients with ventricular septal defect (VSD), one with atrial septal defect (ASD), and two with aortic stenosis were referred for evaluation of hemorrhagic symptoms or an abnormal preoperative hemostatic screening test. Seven of the 12 patients had a history of mucocutaneous bleeding (Table 1). Of these, four patients (3, 6, 8, and 11) had lifelong histories of bleeding; the remaining three were studied at the time of presentation of the symtoms listed in Table 1.

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Table 1. Factor VIII Molecular Complex in Congenital Cardiac Defects

Patient	Cardiac Defect	Bleeding Symptoms	F VIII:C (U/dL)	vW:A (U/dL)	vW:rist (U/dL)	vWF Subunits
1	VSD	Yes*	73	27	23	Abnormal
2	VSD	Yes*	132	140	118	Abnormal
3	VSD, tetralogy of Fallot	Yes†‡	82	65	70	Abnormal
4	VSD	No	83	36	22	Abnormal
5	VSD, coarct (both repaired)	Yes§	145	106	98	Abnormal
6	VSD	Yes*‡‖	49	37	22	Abnormal
7	ASD primum	No	94	45	47	Abnormal
8	VSD	Yes†‡‖	81	43	41	Abnormal
9	VSD	No	54	208	170	Abnormal
10	VSD, coarct (coarct repaired)	No	118	82	62	Abnormal
11	Supravalvular aortic stenosis	Yes†‡¶	101	100	42	Abnormal
12	Aortic stenosis	No	47	42	32	Abnormal

*Excessive bleeding with laceration.

†Easy bruisability or petechiae.

‡Frequent epistaxis.

§Bleeding from tooth extraction for three days.

‖Hematoma at cardiac catheterization site.

¶Gastrointestinal hemorrhage.

Evaluation of the factor VIII molecular complex (Table 1) revealed that all but two of the children had normal concentrations of plasma F VIII:C (50 to 150 U/dL); patients 6 and 12 had borderline abnormal values of 49 and 47 U/dL, respectively. However, analysis of vWF showed that six patients had reduced (<50 U/dL) concentrations of vWF measured both immunologically (vW:Ag) and by ristocetin cofactor assay (vW:rist). Patients 2, 4, 6, 9, 10, 11, and 12 had lower vW:rist values compared to vW:Ag, but there was good agreement between the immunologic and ristocetin cofactor assays in the remaining five patients. All 12 patients had loss of the highest mol wt subunits of vWF demonstrated by SDS-agarose electrophoresis (Fig 1). Low-dose (.6 mg/mL) ristocetin-induced platelet aggregation was absent in two patients (4 and 8); others were not studied. Five patients were studied on two or three occasions over a period of 1 to 18 months prior to surgery. Each patient had repeatedly abnormal vWF multimeric analyses.

The routine coagulation studies are summarized in Table 2. Ten of the 12 patients had a prolonged bleeding time (>10 minutes); in no case was the long bleeding time explained by thrombocytopenia since all twelve patients had a normal or slightly elevated platelet count. With the exception of patient 7, who had a borderline low fibrinogen value, none of the patients had evidence of intravascular consumption of coagulation factors; none had an abnormal PT or thrombin time or elevation of fibrin(ogen) degradation products. Three patients (10, 11, and 12) had a prolonged PTT explained by a decreased factor XII level (14, 44, and 41 U/dL, respectively).

To determine whether the abnormal vWF multimeric pattern was present in association with the cardiac anomaly, five patients were studied after surgical correction of the cardiac defect. Four patients (4, 6, 8, and 9) who had loss of the largest vWF multimers preoperatively had normalization of the multimeric pattern following repair (Fig 2). Although patients 4, 8, and 9 had normal vWF as measured by all methods including vWF multimers and patient 6 had only a mild decrease in both vW:Ag and vW:rist (43 and 45 U/dL, respectively), all four patients still have markedly prolonged bleeding times. Patient 4 had platelet aggregation studies both preoperatively and postoperatively; her platelets aggregated normally when the agonists epinephrine, collagen, arachidonate, and ADP were added to her platelet-rich plasma. The fifth child (patient 5) was studied initially 8 years after repair of her VSD and had abnormal vWF multimers; she also had a residual pressure gradient across a previous pulmonary artery banding site.

Further evidence for the acquired nature of the vWF anomaly was found by family studies. None of the parents available for study had evidence of vWF multimeric abnormalities. Two parents did have mildly decreased concentrations of vW:Ag (36 and 43 U/dL respectively).

To determine if these children were different from others with VSD or ASD who underwent catheterization and surgical repair during the same time period, cardiac catheterization findings were evaluated. Eight of nine patients with VSD had a sealed foramen ovale, and all had high-end

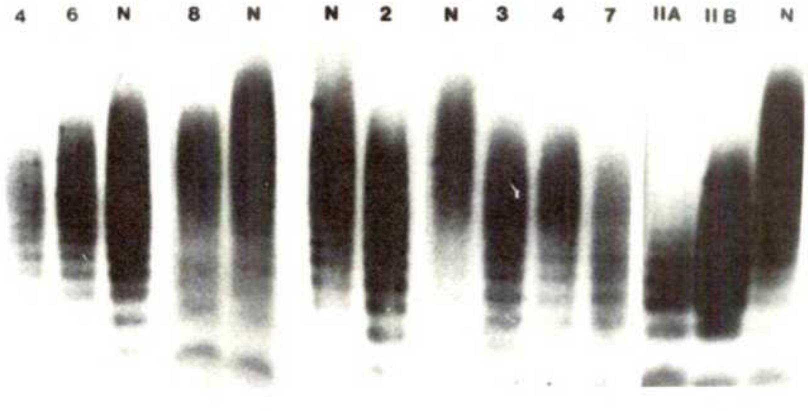

Fig 1. Von Willebrand factor multimers in patients with congenital cardiac defects. N indicates the normal plasma pool. Arabic numerals indicate samples from patients described in the tables. IIA and IIB are multimers from patients with type IIA and IIB vWD for comparison. Note the absence of the highest mol wt multimers in patients with cardiac defects, similar to that seen in Type IIB vWD.

Table 2. Other Coagulation Studies

Patient	Bleeding Time (min)	Platelet Count (U/mm^3)	PT (s)	PTT (s)	TT (s)	Fibrin (ogen) Degradation Products (μg/mL)	Fibrinogen (mg/dL)
Normal	2.5-10.0	150,000-350,000	11.0-12.6	25.0-35.5	12.6-15.2	<10	200-400
1	>20.0	352,000	12.0	30.1	13.0	—	—
2	>20.0	602,000	10.8	25.6	13.5	—	—
3	>20.0	388,000	11.9	30.4	13.5	<10	292
4	>20.0	289,000	11.4	30.6	12.6	<10	255
5	8.5	450,000	12.6	31.9	13.5	—	—
6	17.0	306,000	11.9	33.5	12.5	<10	245
7	16.0	370,000	12.2	27.3	13.5	<10	193
8	>20.0	366,000	12.5	32.5	13.6	<10	325
9	>20.0	312,000	11.9	33.3	13.5	—	270
<10	19.5	363,000	12.7	40.2	—	<10	420
11	>20.0	244,000	12.1	36.2	14.9	—	—
12	9.0	359,000	12.2	35.2*	14.4	<10	277

*This PTT was outside the normal range established at the time of this study.

diastolic pulmonary artery pressure. Since other patients with cardiac defects may also present these features, it is unknown whether this finding is the only explanation for the vWF abnormalities.

One patient (6) was studied before and after infusion of cryoprecipitate. After administration of 1 donor unit/5 kg body weight, his vW:Ag rose from 37 to 60 U/dL and his vW:rist from 22 to 65 U/dL, and his abnormal multimeric pattern was almost completely corrected (Fig 2). His bleeding time, however, only partially corrected from a preinfusion value of 17 minutes to 12 minutes postinfusion. None of the patients had excessive intraoperative or postoperative bleeding, but all received large amounts of fresh frozen plasma and platelet concentrates during the postoperative period as part of the routine surgical management of open-heart cases. No increased use of such products was noted.

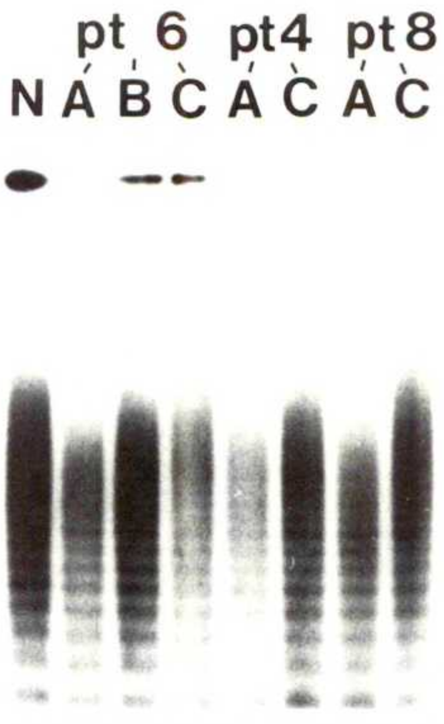

Fig 2. Normalization of vWF multimers in patients with congenital cardiac defects following surgical repair and following infusion of cryoprecipitate. For each patient, lane A contains the preoperative sample and lane C the postoperative sample. Lane B is the postcryoprecipitate infusion sample from patient 6. Note normalization of the vWF multimer pattern in lanes B and C.

DISCUSSION

Von Willebrand factor is a plasma protein composed of large multimers of .4 × 10^6 to 20 × 10^6 daltons mol wt.[3] Both inherited and acquired abnormalities of the protein have been described; patients with a vWF hypoproteinemia (decreased amounts of normal vWF) have von Willebrand disease of type I, and those with a dysproteinemia (abnormal vWF with normal or reduced amount of protein) have type II. Type II vWD is characterized by the absence of the large vWF multimers when analyzed by SDS-agarose electrophoresis. The absence of these hemostatically important multimers in type II vWD results in defective platelet adhesion and a clinically significant bleeding disorder.[1-3]

Acquired type II–like vWF abnormalities have been described in patients with episodic hemolytic uremic syndrome,[6] DIC,[4,5] myeloproliferative syndrome,[8] and acute myocardial infarction,[7] and we have seen this in one case of acquired type II vWD (Scott and Montgomery, unpublished observation). These patients exhibit an absence of the largest plasma vWF multimers and a discrepancy between vWF measurements by antigenic and functional assays. Our 12 patients with congenital cardiac defects also appear to have a form of acquired vWD; they have loss of the largest vWF multimers, long bleeding times, histories of mild hemorrhagic episodes, and, in one patient studied, a prolonged bleeding time that was partially corrected by the administration of cryoprecipitate. It is uncertain if all symptoms are related to the vWF defect, however, since four patients have residual hemostatic abnormalities (prolonged bleeding times) even though their vWF multimers have become normal following surgical repair of the cardiac defect. The acquired nature of the phenomenon is demonstrated by the correction of the vWF defect with surgical correction of the cardiac defect in four of the patients studied both preoperatively and postoperatively and the lack of the abnormality in parents of the affected patients. The one patient with persistence of the abnormality after correction of her VSD had an abnormal pressure gradient across a previous pulmonary artery banding site; the vWF abnormality might be explained by persistence of an abnormal hemodynamic state in this patient

The mechanism of loss of the largest vWF multimers associated with these congenital cardiac defects is unclear. Review of cardiac catheterization data in the 12 patients failed to reveal a specific characteristic that was different from those found in other children with similar cardiac lesions, with the exception that all but one of the patients with VSD had a sealed foramen ovale. Perhaps the abnormal hemodynamic flow is associated with activation of platelets or endothelial cells with resultant adsorption of the largest vWF multimers from the plasma. If adsorption of vWF to platelets is responsible for its absence from plasma, one might expect that the platelets would be agglutinated by low-dose ristocetin as is seen in type IIB vWD;[3] this was not present in the two patients tested (patients 4 and 8), although the multimeric pattern on electrophoresis in SDS-agarose is more reminiscent of type IIB vWD than of type IIA, in which all of the high–mol wt multimers are absent, rather than only the largest as seen in these patients. Another possible mechanism for the phenomenon is cleavage of the vWF multimers into smaller forms by proteolytic enzymes (possibly by plasmin[13–15] and/or the calcium-activated protease[16] similar to that described in patients with DIC[4,5]. However, there was no evidence of DIC in these patients; all had a normal PT, thrombin time, and platelet count and, in the eight studied (patients 1, 3, 4, 6, 7, 8, 10, and 11), normal concentrations of fibrinogen with lack of fibrin(ogen) degradation products. The three patients with prolonged PTTs had concurrent factor XII deficiency; none has as yet undergone surgical correction of their cardiac lesion. An association of factor XII deficiency with vWD has been reported previously;[17,18] the significance in these patients is unknown

The variety of hemostatic defects associated with congenital cardiac lesions manifested by thrombocytopenia, platelet dysfunction, and deficiency of clotting factors because of decreased synthesis, fibrinolysis, and DIC appears to be directly related to the hypoxia and hyperviscosity associated with cyanotic congenital heart disease.[19] However, as described here, in 7 of 12 children with noncyanotic cardiac lesions, clinically significant bleeding histories were found. All 12 had an associated alteration in von Willebrand factor multimers. However, it is not clear that the von Willebrand factor abnormality contributed to the bleeding histories or prolonged bleeding times since the bleeding times were not corrected with normalization of vWF following corrective cardiac surgery. Further studies to elucidate the mechanism of this phenomenon and to determine its prevalence and clinical significance in VSD and other cardiac lesions are ongoing in our laboratory.

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The Sensitivity to Cytosine Arabinoside of the Blast Progenitors of Acute Myeloblastic Leukemia

By Nobuo Nara, J.E. Curtis, J.S. Senn, D.L. Tritchler, and E.A. McCulloch

Two culture methods are available for the study of the blast cells of acute myeloblastic leukemia (AML). One is an assay for clonogenic precursors; it depends on their ability to form blast colonies in culture in the presence of methylcellulose and suitable growth factors. The other assesses the growth of blast cells in suspension culture, where growth is measured by increasing numbers of clonogenic cells. We have compared the two methods as assays for the cytotoxic effects of the chemotherapeutic drug cytosine arabinoside (Ara-C). Marked patient-to-patient variation was found using either method; however, the slopes of the dose-response curves were usually greater when cells were exposed to drug in suspension rather than in methylcellulose. Control experiments showed that the difference could not be explained by drug carry-over from the suspension cultures to the methylcellulose plates when clonogenic cells in the suspensions were assessed. Further, the survival curves for Adriamycin were very similar, regardless of which assay was used. No correlation was found between D_{10} Ara-C values measured in suspension or in methylcellulose. However, a significant association with outcome was found between D_{10} Ara-C in suspension and response to treatment with a regimen in which Ara-C was the only chemotherapeutic agent used. No such association was detected when the D_{10} values obtained with the clonogenic assay were compared with outcome for the same group of 15 patients. Finally, a feasibility experiment was performed in which blast cells were exposed to Ara-C repeatedly during exponential growth over 238 days. A dose-related inhibition of growth was observed; no evidence was seen of emerging drug-resistant cells. Nor did the morphology of the cells change as a result of drug exposure. We conclude that drug sensitivities of AML blast cells in culture are dependent on measurement methods, even when techniques affecting cell proliferation are compared. Measurements of drug sensitivity in culture may best be interpreted when the bases of the assay systems are understood.

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THE RESPONSE TO chemotherapy provides striking evidence of heterogeneity among patients with acute myeloblastic leukemia (AML). Some die before adequate treatment can be delivered Many, but not all, treated patients enter remission. Of these, most relapse within a year, and only a few survive for 5 years.[1-3] It is a research goal to understand the basis of this heterogeneity. We have approached the problem by seeking associations between outcome following chemotherapy and various attributes of patients, treatment regimens, or leukemic cells. For these last, cell culture methods have proved useful in the characterization.

We now employ two techniques for the culture of blasts. The first is a clonogenic assay: A small proportion of the blast population will form colonies in cultures made viscid by methylcellulose, provided a suitable source of growth factors is included.[4,5] In the second method, blast progenitors multiply exponentially in suspension culture; the requirements for growth are a cell density of 10^6 cells/mL and the same source of growth factors used in the clonogenic assay.[6]

Both methods are consistent with a model of the blast population that postulates its maintenance by blast stem cells; these may renew themselves or enter a determination-like process yielding cells capable of only terminal divisions.[7] In the clonogenic assay, colony formation reflects primarily these terminal divisions; self-renewal can be demonstrated only by replating colonies, either singly or as pools.[8] This secondary plating efficiency (PE_2), though variable from patient to patient, is always low, indicating that few of the divisions required for colony formation lead to new clonogenic cells. On the other hand, the exponential increase in blast progenitors seen in suspension cultures may be considered to reflect self-renewal; the presence of end cells is deduced from the observation that the doubling time in culture of blast progenitors growing in suspension is longer than their generation time. The difference in the extent of self-renewal seen in the two assays may reflect inhibitory effects of methylcellulose.[6]

Blast heterogeneity is evident with either culture method. For the clonogenic assay, a significant association has been found repeatedly between successful remission induction and low plating efficiencies when blast colonies are subcultured (PE_2).[8-10] In contrast, no such link has emerged between response and the sensitivity of colony formation to the chemotherapeutic drugs used in treatment. Others have obtained similar results, particularly in settings where cytosine arabinoside (Ara-C) was the only chemotherapeutic agent used.[11,12] It seemed possible that the effect of drug on self-renewal might be more important than its capacity to reduce colony formation. This view was strengthened with the finding that PE_2 was reduced compared to controls when colonies surviving after exposure to Ara-C were replated,[9,13] while no such change was found after exposure to Adriamycin.

These considerations led us to compare the Ara-C sensitivity of blasts when determined by each method. We found marked patient-to-patient variation regardless of technique; however, Ara-C was usually more effective against blasts in suspension than in methylcellulose. Further, the slopes of the simple negative exponential survival curves (D_{10}, dose required to reduce survival to 10% of control) obtained by

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each technique were not correlated, and for a small number of patients, a significant association was found between D_{10} Ara-C in suspension and successful remission induction. We also demonstrated the feasibility of exposing blast populations to drug repeatedly during prolonged exponential growth.

MATERIALS AND METHODS

Patients and treatment Heparinized peripheral blood was obtained from 21 patients with AML at the time of diagnosis or at relapse, with informed consent, as prescribed by a protocol approved by the Human Experimentation Committee of the University of Toronto. The diagnosis was based on cell morphology according to the FAB classification[14] and the clinical criteria used at the Ontario Cancer Institute in clinical trials of AML.[15] Table 1 contains the clinical characteristics, treatment, and responses to treatment of the patient group. The patients seen at diagnosis received a high-dose Ara-C protocol [16] The protocol consists of Ara-C 3 g/m^2 of body surface area (BSA) twice daily for six days (reducing to 2 g/m^2 BSA in patients $\geq$ 60 years) and prednisone 40 mg/m^2 BSA daily for six days for remission induction. One patient (No 6) refused treatment. Patient 5 had a previous neoplasm (carcinoma of lung) These two patients were excluded from the assessment of the reponse to therapy The patients seen at relapse were treated with a combination of chemotherapeutic drugs.

Patients were considered to have responses if they had achieved either a complete remission (CR) or a partial remission (PR).[15] The criteria for complete remission included a marrow of approximately normal cellularity with <5% blasts and <5% promyelocytes, normal number of megakaryocytes, and evidence of regenerating granulopoiesis and erythropoiesis. Normal peripheral blood counts and no evidence of clinical disease elsewhere were also required. A partial remission required a marrow of normal or moderately increased cellularity with >10% of blasts and promyelocytes but <25% blasts present Patients were followed by examinations of the peripheral blood; in all instances, the blast population was eliminated. Early (14-day) marrow aspirations were not done routinely since, in our hands, this procedure has not proved useful clinically. Bone marrow was aspirated to assess response to treatment when normal leukocytes or blast cells began to appear in the peripheral blood. This usually occurred 4 to 5 weeks after treatment was started

Blast colony assay. Primary colony formation was obtained as previously reported,[4,5] except that supernatants from cultures of the continuous line of bladder cancer cells, HTB9, obtained from the American-type culture collection, were used rather than media conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM).[17] Suspensions containing 4 × 10^5 T cell–depleted mononuclear cells from peripheral blood suspended in 1 mL of α-MEM supplemented with 20% fetal calf serum (FCS), 10% HTB9-conditioned medium, and .8% methylcellulose were cultured in 35-mm Lux culture dishes (Miles Laboratories, Naperville, Ill) for seven days at 37° C and 5% CO_2 in air. Colonies with more than 20 cells were scored, and plating efficiency was described as PE_1. Four replicate plates were used to determine PE_1 for each assay.

Replating from methylcellulose Blast progenitors growing in methylcellulose were replated as described previously,[8] except for the substitution of HTB9-conditioned medium for PHA-LCM. Cell suspensions prepared from primary blast colonies in methylcellulose were washed twice in α-MEM and then replated at a concentration of 10^4 cells in .1 mL in Linbro microwells (Flow Laboratories, McLean, Va) of α-MEM with 20% FCS, 10% HTB9-conditioned medium, and .8% methylcellulose Colonies with more than 20 cells were scored after seven days' incubation, and their plating efficiency was designated as PE_2. Four replicate wells were used to determine PE_2. Secondary colonies have been shown to be similar to primaries in terms of growth requirements and cellular morphology by light and electron microscopy.[18]

Table 1. Clinical Data

Patient	Diagnosis (FAB)	Age	Peripheral Blood Blasts per µL	Treatment*	Outcome†
Patients at diagnosis					
1	M2	29	5,400	HD-Ara-C	PR
2	M4	39	25,600	HD-Ara-C	CR
3	M2	43	62,264	HD-Ara-C	F
4	M4	60	342	HD-Ara-C	CR
5	M4	56	1,326	HD-Ara-C	PC
6	M2	68	36,720	None	NA
7	M2	62	38,136	HD-Ara-C	F
8	M1	36	432	HD-Ara-C	CR
9	M2	20	1,938	HD-Ara-C	F
10	M1	38	3,264	HD-Ara-C	CR
11	M4	36	11,557	HD-Ara-C	CR
12	M2	71	46,872	HD-Ara-C	PR
13	M1	52	3,087	HD-Ara-C	F
14	M4	51	56,425	HD-Ara-C	CR
15	M4	69	54,340	HD-Ara-C	F
16	M1	60	46,514	HD-Ara-C	CR
17	M4	27	3,706	HD-Ara-C	F
Patients in relapse					
18	M4	74	6,059	ACT	F
19	M4	73	3,484	None	NA
20	M2	70	360	L-asp, MTX	F
21	M1	39	33,888	Ara-C, L-asp	PR

*HD-Ara-C, high-dose cytosine arabinoside; ACT, Adriamycin, Ara-C, and 6-thioguanine[15], L-asp, L-asparaginase, MTX, methotrexate.

†PR, partial remission; CR, complete remission; F, failure; PC, previous cancer (not included in assessment; see Materials and Methods, NA, not applicable

Liquid suspension culture. Blast progenitors were cultured in liquid suspension as described previously,[6] modified by the substitution of HTB9-conditioned medium for PHA-LCM. T cell–depleted mononuclear cells from peripheral blood at a concentration of 10^6 mL were suspended in 3 mL of α-MEM supplemented with 20% FCS and 10% media conditioned by HTB9 cells The blasts were cultured in suspension in 35-mm Lux tissue culture dishes at 37° C in a humidified atmosphere of 5% CO_2 in air. Cultured cells were harvested and counted at day 7 Primary and secondary plating efficiencies (PE_1 and PE_2) were determined using the methylcellulose culture method described above.

Assessment of blast progenitor growth in suspension. The growth of clonogenic blasts in suspension was assessed by combining the methylcellulose assay with the suspension culture assay. After a period of time in suspension (usually seven days), the cells were harvested, washed, counted, and plated in methylcellulose to obtain the plating efficiency By multiplying the number of cells recovered from each dish by this plating efficiency, the number of clonogenic cells per dish was calculated. This value could be compared to the input of nucleated cells (see Figure 2, for example). For experiments where cells were replated serially, the dilution at each subculture could be used to calculate the cumulative growth of clonogenic blast cells with time (for an example, see Fig 8).

Sensitivity to Ara-C. Figure 1 illustrates the method used to measure the sensitivity of blast progenitors to Ara-C in suspension or in methylcellulose. Blast cells were placed in either culture condition

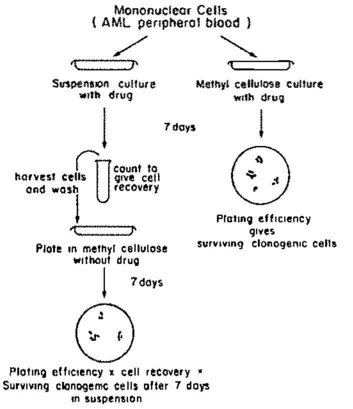
Mononuclear Cells
(AML peripheral blood)
Suspension culture with drug
Methyl cellulose culture with drug
7 days
harvest cells and wash
count to give cell recovery
Plating efficiency gives surviving clonogenic cells
Plate in methyl cellulose without drug
7 days
Plating efficiency x cell recovery = Surviving clonogenic cells after 7 days in suspension

Fig 1. How drug dose-response curves are obtained for AML blast progenitors exposed to drug for seven days in either suspension culture or methylcellulose culture.

for seven days in the presence of increasing concentrations of Ara-C. At the end of the period, the suspension cultures were harvested, washed twice in a-MEM, and assayed for colony formation in methylcellulose in the absence of Ara-C. The recovery of clonogenic cells per dish was calculated as described above, and its decrease with increasing exposure to Ara-C in suspension was used to construct survival curves After seven days, the colonies in the methylcellulose cultures in the presence of drug were counted; the plating efficiency decreased as a function of drug dose, as described previously.[19,20] Thus two survival curves were obtained for the same cell population exposed to Ara-C for the same time period, but either in suspension or in methylcellulose

Sensitivity to Adriamycin. Similarly, the sensitivity of blast progenitors to Adriamycin was measured in suspension or methylcellulose cultures.[21] Two regimens were used In the first, the cells were exposed to varying concentrations of drug for ten minutes, washed, and cultured either in methylcellulose or in suspension. In the second, the cells were exposed continuously to the adriamycin during seven days' growth in methylcellulose or suspension cultures. In the latter instance, the cultures were washed before replating the cells in methylcellulose in the absence of Adriamycin

Cellular phenotypes Cellular phenotypes were determined by using several monoclonal antibodies. Among them, OKM-1,[22] 8OH.5,[23] and My-1[24] were used as reagents specific to granulopoietic cells. MO-2[25] was used as a reagent specific to monocytes.

Statistics. Group data were compared by Student's t test D_{10} values for Ara-C and Adriamycin were determined from the slope of negative exponential dose-response curves calculated by linear regression Dose-response curves were compared as described by Gaddum.[26] Data are presented as the mean ± SE. Jonckheere's test was done as described in Lehmann.[27]

RESULTS

Ara-C survival curves. Quantitative measurement of survival curves depends on the relationship between the number of cells added to cultures and the outcome of the procedure. Figure 2, representative of two experiments using samples containing sufficient blasts, shows this relationship to be linear for the number of blasts cells used to initiate suspension cultures and the recovery of clonogenic cells after seven days ($r = .971$, $P < .01$). Further, the presence of Ara-C in the cultures, while decreasing the yield of clonogenic blasts, did not alter the linearity of the relationship between blast input and clonogenic blast recovery ($P < .05$).

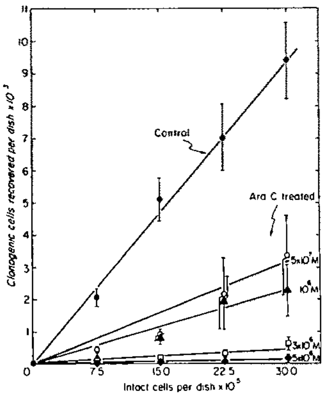

Fig 2. The relationship between the input intact cell numbers and the recovery of clonogenic cells after seven days of suspension culture in the absence of Ara-C (●) or presence of Ara-C at the doses of 5 × 10^{-7} (○), 1 × 10^{-6} (▲), 3 × 10^{-6} (□), and 5 × 10^{-6} mol/L (◆). Autologous irradiated cells were added to provide a fixed number of cells (3 × 10^{-5} per dish). The relationship is not significantly different from linear in the control and at all concentrations of Ara-C.

A typical Ara-C survival curve for blasts in suspension is shown in Fig 3. The simple negative exponential form of the curve is apparent. The survival curve obtained using the clonogenic assay, as described in Materials and Methods, is included in the figure. As reported previously,[19,20] such survival curves are also simple negative exponentials. Both results can therefore be described by a single parameter, the D_{10}. It is evident in Fig 3 that the D_{10} value was much greater for the clonogenic assay than for the suspension culture procedure.

The D_{10} values obtained for both methods are compared in

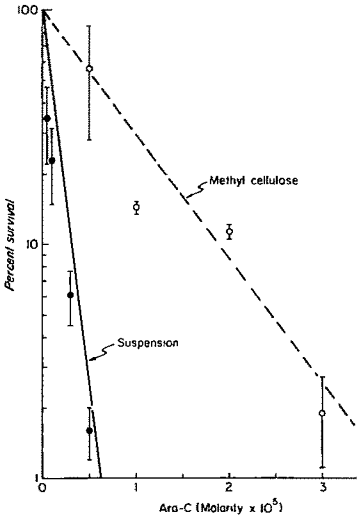

Fig 3. Ara-C survival curves for blasts in suspension (●) or methylcellulose (○) culture. Survival curves are simple negative exponentials. The curves may be described by the D_{10} value, the concentration of drug required to reduce survival to 10% of control.

Fig 4 No significant correlation was found between the two measurements ($r = .249$, $P > .25$) It is evident that the Ara-C survival curve measured in suspension was usually less than that obtained using the clonogenic assay. In one instance, patient 17, D_{10} in suspension was slightly greater than D_{10} in methylcellulose

Drug carry-over control. In the suspension culture methods, Ara-C is added to cultures at varying concentrations; after seven days, the cells are washed and replated in methylcellulose. It is possible, therefore, that Ara-C itself or an active metabolite might be carried from the suspension cultures into the methylcellulose assay plates. The action of such carried-over drug might explain the marked sensitivity observed with the suspension culture method.

Two experimental designs were used to test for this possibility. In the first, blast cells were exposed to Ara-C for seven days, recovered, washed, and irradiated to destroy their proliferative capacity. These were then added to varying numbers of intact cells, and the mixture was plated in methylcellulose. Colony formation as a function of the number of intact cells plated is shown in Fig 5A. It is evident that the plating efficiencies of clonogenic blasts in methylcellulose had not decreased when plated in the presence of cells that had been exposed to Ara-C under the conditions used to measure drug sensitivity in suspension.

In the second control design, irradiated cells obtained from cultures with or without Ara-C were added to varying numbers of intact cells, and the mixtures were cultured in suspension. Figure 5B shows the results obtained when the cells were tested for clonogenic blasts after seven days. The same linear relationship between input viable blasts and recovery of clonogenic blasts was maintained regardless of whether the irradiated supporting cells had been exposed to Ara-C or not.

These two experiments make carry-over of Ara-C an unlikely explanation for the differences in Ara-C survival curves seen when blasts were tested in suspension or in methylcellulose. In addition, the second design shows that exposure of blasts to Ara-C does not destroy their capacity to support the growth of small numbers of intact cells in suspension

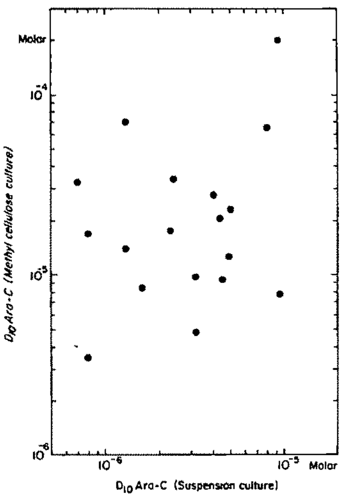

Fig 4. Relationship between D_{10} values in suspension and in methylcellulose cultures. There is no significant correlation ($P > .2$) r = 0.249.

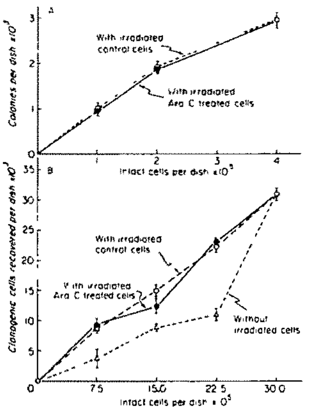

Fig 5. Drug carry-over experiments. Blast cells were exposed to Ara-C at the D_{10} dose for seven days in suspension culture. Then they were harvested, washed, and irradiated (20 Gy). They were added to intact blast cells in methylcellulose (A) or suspension culture (B) to provide a fixed cell number. As a control, blasts incubated in the absence of Ara-C were added. There was no significant difference between the effects of Ara-C-treated cells (●) and nontreated cells (○)

Adriamycin sensitivity. The comparison between Ara-C survival curves using two different methods is based on the assumption that both examine the same proliferative entity The requirement for a single growth unit in each is supported by the linear relationship between cells added to cultures and response shown for each method in Fig 2 and 5. Evidence has been presented that the two procedures display different properties of the same cell.[6] However, the possibility remains that a cell more primitive than the clonogenic blast detected in methylcellulose is growing in suspension and then undergoes a transition to a clonogenic blast when placed in methylcellulose. The existence of such a blast progenitor might explain the different Ara-C survival curves found in suspension and in methylcellulose. If this were the explanation, survival curves for a different drug might be expected to show the same phenomenon. Adriamycin is a candidate since marked patient-to-patient variation is found when Adriamycin survival curves are measured. Further, Adriamycin differs from Ara-C in its effects on the composition of colonies surviving drug exposure. Colonies in Ara-C-treated plates often contain fewer new clonogenic cells than controls;[9] in contrast, the PE_2 values for colonies developing after Adriamycin treatment are similar to control values. These data have been interpreted as indicating that cell-cycle processes required for self-renewal might be more sensitive to Ara-C than those that serve cell division generally.[13]

Blast progenitor survival curves for Adriamycin are shown in Fig 6 In the experiment, either cell suspensions were exposed briefly to drug, or Adriamycin was included in the culture medium.[21] It is apparent that much greater sensitivities were found with the latter procedure However, regardless of the length of exposure of cells to Adriamycin, very

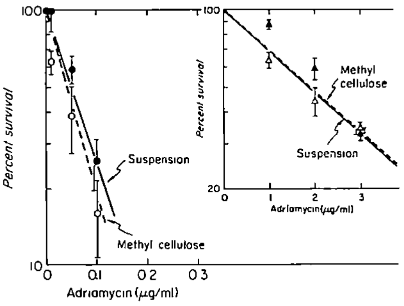

Fig 6. Adriamycin dose survival curves. Adriamycin was added to suspension culture and methylcellulose culture. Survival curves are simple negative exponentials. The insert shows the effect of brief exposure (ten minutes) to Adriamycin. Survival curves in suspension and in methylcellulose were indistinguishable.

similar D_{10} values were obtained both with the methylcellulose procedure and after growth in suspension. This finding supports the view that the growth unit is the same or very similar in both the suspension and methylcellulose assays and strengthens the basis for comparing survival curves obtained by the two methods.

Association with outcome. In our previous studies, D_{10} Ara-C measured in methylcellulose was not an attribute contributing to variation in outcome.[10] This conclusion was based on clinical data from patients treated with multidrug regimens and using the assay for colony formation in methylcellulose. Although sensitivity data are available for only 15 patients treated with high-dose Ara-C at the time of first diagnosis, we undertook to compare the association of successful remission induction with D_{10} values measured either in suspension or in methylcellulose (Fig 7). When we followed our usual practice (see Materials and Methods) by including in the analysis all patients who received at least one dose of drug and by considering partial remissions as responses, a significant association ($P < .01$) (see Materials and Methods) was found for D_{10} values in suspension but not in the 14 samples assayed in methylcellulose ($P > .2$). Additional analyses were done after removal from the data set of patients who died within the first 48 hours of treatment. In the first of these analyses partial remissions were not included; significant association was maintained between low D_{10} values in suspension culture and successful remission induction ($P < .05$) but not for the methylcellulose assay ($P > .2$). Only if the two patients with partial remission were considered as failures did the association of D_{10} value in suspension with remission induction lose significance. It seems inappropriate to add the partially responding patients to the failure group, since, following treatment with Ara-C, blasts were reduced in these patients and normal hematopoiesis improved It may be more useful to group the patients is three sets: remission, partial remission, and failure (excluding early deaths) Then the D_{10} values for each set can be compared using Jonckheere's test,[27] which is the appropriate statistic to determine if sets are ordered in ascending values of D_{10} (that is, D_{10} CR < D_{10} PR < D_{10} failure). This analysis showed that such ranking is significant for these data ($P < .05$). This finding is consistent with the hypothesis that increasing D_{10} values in suspension are associated with decreasing probability of responding to treatment. Jonckheere's test did not give significant P values when applied to the D_{10} values measured in methylcellulose.

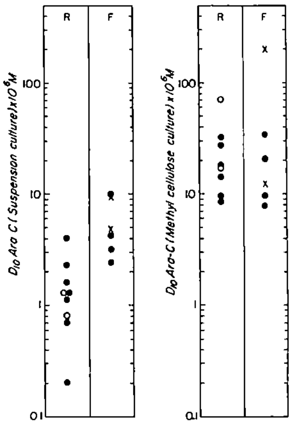

Fig 7. Comparison of patients who responded (R; completely, ●; partially, O) or failed (F) treatment with high-dose Ara-C administered at diagnosis. Early deaths are shown as X. The D_{10} values are shown on the horizontal axis. The two columns at the left contain the results obtained using the suspension culture method. The columns at the right contain the values obtained using the methylcellulose assay. See the text for a description of the analysis.

We considered other factors that have been found to be associated with outcome. In Table 2, age, number of blasts per milliliter in the peripheral blood, platelet count, and plating efficiencies in methylcellulose (PE_1 and PE_2) are compared with D_{10} Ara-C in suspension; the results of testing these attributes for an association with successful remission induction are included in the table. As in previous studies,[8,10] a significant association was found between PE_2 and response; PE_2 was also correlated with D_{10} Ara-C in suspension. None of the other characteristics of patients or of their disease were associated with outcome of treatment or with D_{10} Ara-C in suspension. These patients were all treated with large doses of Ara-C (see Materials and Methods); not surprisingly, blasts were cleared from the peripheral blood in all patients who received a full initial course of treatment, including those who failed to respond (Table 3). It may be, therefore, that the association between low D_{10} Ara-C values, measured in suspension, and successful remission induction is based on some drug action in addition to general cytotoxicity.

Repeated exposures to Ara-C. A major advantage of the suspension culture is that some blast populations may be

Table 2. Statistical Associations of Attributes

	Association With Remission Induction	Association With D_{10} Ara-C in Suspension Culture	
	P	r	P
Age	> 1	.030	>.25
Blasts in blood	>.1	.272	> 1
Platelets	>.1	.002	> 25
PE_1	> 1	.135	> 25
PE_2	< 01	.503	< 05

Table 3. Failure Group

Patient No	Aplasia of Bone Marrow	Clearance of Blasts From Blood	Blast Regrowth Bone Marrow	Blast Regrowth Blood	Outcome
3	ND	NA	ND	NA	Died at 1 day
7	no	yes	ND	no	Died at 1 month
9	yes	yes	yes	yes	Died at 1 year
13	yes	yes	yes	no	Alive at 4 months
15	ND	NA	ND	NA	Died at 1 day
17	ND	yes	ND	yes	Died at 2 months

NA, not applicable; ND, not determined.

*Blasts were predominant in a cellular bone marrow 4 weeks after treatment, but no further assessment was conducted before death

maintained in the culture for periods of weeks. This permits the examination of the effects of repeated exposures to chemotherapeutic agents, a setting that mimics the clinical use of drugs. Figure 8 displays the results of an experiment in which blast cells were exposed to Ara-C repeatedly over a period of 238 days. Two doses were used, 10^{-7} and 10^{-6} mol/L; the Ara-C was added at the time of subculture, and the cells were not washed until the next scheduled change of medium, 1 week later. Thus the drug exposure schedules were the same as those used to measure Ara-C survival curves

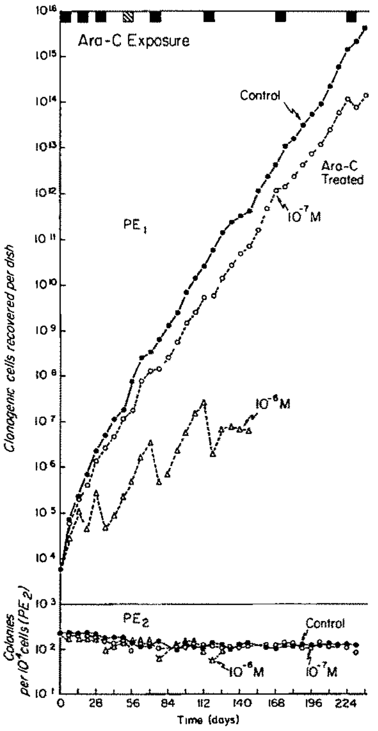

Fig 8. Repeated exposure of blasts from patient 18 during prolonged culture in suspension. The cells were grown for seven days, counted, washed, diluted to a concentration of 10^6/mL, and recultured. The curves in the top panel of the figure were constructed by accumulating the growth at each subculture. Control cultures are shown as ●——●. Cells were exposed to Ara-C at the times shown at the top of the figure. Those exposed to 10^{-7} mol/L (O——O) Ara-C were treated at each time indicated. Those exposed to 10^{-6} mol/L (Δ——Δ) did not receive drug at the time indicated by ▨ since recovery was not then sufficient. The PE_2 values for the three groups are shown in the bottom panel, using the same symbols as in the top panel.

It is evident that the control cultures grew exponentially, with a doubling time of between three and four days At the lower drug dose, growth was inhibited immediately following exposure but resumed after the next subculture. In the cultures that received Ara-C at the 10^{-6} mol/L dose, a fall in clonogenic cells was seen after each exposure, followed by a resumption of growth after the cells were washed and resuspended.

We looked for changes in blast cell properties following repeated drug exposure. At the high dose, the degree of fall in clonogenic blasts was similar following each exposure, indicating that sensitivity to Ara-C had not changed. Sufficient cells were available from the control cultures and those exposed to 10^{-7} mol/L Ara-C to permit the construction of detailed survival curves. After 96 days in culture, the D_{10} values were 2.4×10^{-6} and 3.4×10^{-6} mol/L for the control and drug-exposed cultures, respectively. These values are not significantly different ($P > .05$). At day 189, the blast progenitors remained Ara-C-sensitive, with a D_{10} for the 10^7 mol/L Ara-C-treated cultures of 1.5×10^{-6} mol/L. The morphology of the cells remained unchanged throughout; in excess of 70% were typical blast cells; the remainder showed some granulation and occasional segmented forms were seen. The predominant immunologically defined markers on the blasts were those associated with granulopoietic cells and macrophages (OKM-1, 8OH.5, My-1, and MO-2); their frequencies did not change during long-term culture, nor were there differences in morphology or marker-defined phenotype between control and Ara-C-treated blast populations.

After 10 weeks in culture, the growth requirements were retested; the need for HTB9-conditioned medium and a cell density of 10^6 cells/mL had not changed. We conclude that in this experiment, repeated exposures to Ara-C did not lead to drug resistance or other phenotypic changes in the blast population.

DISCUSSION

We have shown that the dose-response curve for the effect of Ara-C on AML blasts is dependent on the method used to measure it. Blasts exposed to drug in suspension culture were usually more sensitive than when Ara-C was present during colony formation in methylcellulose. This difference may relfect the mechanism's underlying growth in suspension on the one hand and colony formation on the other. For the former, data are available consistent with the view that growth of clonogenic blasts in suspension reflects their self-renewal rather than recruitment from another population.[6] In contrast, the scarcity of new clonogenic cells in blast colonies has been interpreted to mean that terminal divisions predominate during colony formation.

The finding of uniform sensitivity to Adriamycin in suspension and methylcellulose makes it unlikely that methylcellulose protects cells against drugs by acting as a barrier. Rather, the different effects of culture conditions on the dose-response curves for Ara-C and Adriamycin may reflect their actions on cells; Adriamycin may reduce cell proliferation generally, while Ara-C may have additional toxicity for genetic mechanisms required during self-renewal. From this view point, Adriamycin dose-response curves might be expected to be similar in any assay that depended on cell proliferation, while Ara-C might appear more toxic in an assay that reflects self-renewal.

This hypothesis may be considered in light of the associations of blast properties with remission induction. Low values of PE_2 have been correlated repeatedly with successful remission induction.[8,9,16] Although the small number of patients available for analysis limits the power of the statistical tests, an association is emerging between low Ara-C D_{10} values in suspension and success of treatment with Ara-C. In contrast, for the same series of patients, analyzed by the same statistical methods, no association with response was found when drug sensitivity was measured using colony formation in methylcellulose as an assay. It seems unlikely that drug cytotoxicity in itself would explain an association between response and sensitivity in suspension but not in methylcellulose. The doses of Ara-C adminstered to the patients were high enough to ensure that blasts were cleared from the peripheral blood even in those failing treatment, making it improbable that insufficient cell kill explained failure to achieve remission in the four failing patients who received adequate courses of Ara-C. Rather, sensitivity in suspension may improve response not only through general cytotoxicity but also by interacting with some other component of blast cell function. It is attractive to suggest that this function might be self-renewal. Then the associations with successful treatment of both low PE_2 and sensitivity to Ara-C in suspension might have a similar basis. A large prospective study is needed to evaluate the contributions of PE_2 and Ara-C sensitivity in suspension, using multivariate methods.

We have demonstrated the feasibility of exposing blast cells to chemotherapy repeatedly. The data of Fig 8 support a simple cytotoxic model of chemotherapy. At low doses, the doubling time of the blasts was prolonged; at a high dose, reduction of clonogenic blasts was seen after each exposure, followed by regrowth and eventually cessation of proliferation. For this dose, the extent of the reduction was similar after each exposure, indicating that Ara-C sensitivity had not changed. At the lower dose, sufficient cells were available to construct survival curves. The D_{10} values were not significantly different ($P > .05$). Thus we did not obtain evidence of the emergence of drug-resistant cells. The morphology and immunologically defined phenotypes of the blast population remained similar in the treated and control groups. No evidence of increased differentiation was seen It is noted, however, that this experiment, though requiring a massive effort over 238 days, is but a single example. It needs to be repeated with other blast populations and using other schedules. Our results show only that cells in culture can be used to model the repeated drug exposures used in treatment.

Finally, a major conclusion from these data has certain consequences for the assessment of chemotherapy using cell culture methods. It is evident that the dose-response curve for any agent may be dependent on the method used to determine it, even when techniques are compared that reflect drug effects on cell proliferation. It follows that the use of such dose-response curves, whether for predicting outcome of treatment or selecting drugs, will be most effective if the mechanisms underlying the assay procedures are understood.

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Bone Marrow Transplantation in Patients Aged 45 Years and Older

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Increasing age has been reported to be a poor prognostic factor for survival after bone marrow transplantation. We evaluated causes of death and frequency and type of complications after marrow grafting in 24 syngeneic and 39 allogeneic recipients who were 45 to 68 years old at the time of transplant. Most patients were in an advanced stage of hematologic malignancy. Among patients given syngeneic transplants, actuarial disease-free survival at 7 years is 20%. The major causes of death were relapse of leukemia and idiopathic interstitial pneumonia. Among allogeneic recipients, 9 (23%) are currently alive, and actuarial disease-free survival at 7 years is 11%. Cytomegalovirus pneumonia and septicemia were the most frequent causes of death. Patients over 50 years of age had the poorest survival rate (1/13), but many of these were transplanted in an advanced stage of their disease. However, among 12 patients transplanted while in remission or at an early stage of their disease, 5 are surviving 65 to 1,160 days after transplantation, with an actuarial survival rate of 22% at 3 years. This is in contrast to those who received their transplant in relapse: 2 out of 20 patients (10%) became long-term survivors, with a probability of survival of 15% at 3 years.

The actuarial incidence of grade II through IV acute graft-*v*-host disease (GVHD) was 30% for allogeneic recipients 45 to 50 years of age. This was not significantly different from the incidence in younger patients. In patients 51 to 62 years of age, the actuarial incidence of acute GVHD was 79%; however, this group included three partially HLA-mismatched transplants. Ten of 15 patients surviving at least 3 months developed chronic GVHD.

These results suggest that marrow transplantation is feasible and should be considered in patients over 45 years, especially if recipients are in good clinical condition and are at an early stage of their disease, such as the chronic phase of chronic myelogenous leukemia and preleukemia. For patients more than 50 years of age, allogeneic marrow grafting cannot presently be considered first-line therapy.
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HIGH-DOSE CHEMORADIOTHERAPY followed by marrow transplantation is an effective therapeutic approach for patients with aplastic anemia and hematologic malignancies. About 75% of patients with aplastic anemia and 50% with acute (AML) or chronic myeloid leukemia (CML) can apparently be cured if marrow grafting is performed early in the course of the disease.[1–7] Many centers, however, will not accept patients over 40 or 45 years of age for this form of therapy because increasing age has been reported to be associated with an increased incidence of morbidity and mortality after marrow transplantation.[8–11]

The purpose of the present retrospective analysis was to determine the spectrum and frequency of transplant-related complications and the survival rate and causes of death in patients 45 years and older who received an allogeneic or syngeneic marrow graft in Seattle after 1977. Studies with intensive conventional chemotherapy in acute myeloid leukemia have shown that patients over 60 years of age are able to tolerate intensive chemotherapy.[12–14] The data from our study indicate that older patients can benefit from bone marrow transplantation as well, especially for CML in the chronic phase and preleukemia. However, in patients more than 50 years of age, the incidence of transplantation-related complications such as interstitial pneumonia and acute graft-*v*-host disease (GVHD) increases significantly.

MATERIALS AND METHODS

Between January 1, 1978, and December 1, 1984, 56 patients who were 45 to 68 years of age underwent marrow transplantation for hematologic malignancy, 4 for aplastic anemia, and 3 for preleukemia Patient characteristics are summarized in Table 1. Twenty-four patients received marrow from a monozygotic (syngeneic) twin donor, and 39 received allogeneic marrow from an HLA-identical (n = 34) or HLA-partially incompatible sibling or other relative (n = 4). One patient (unique patient number [UPN] 2588) was transplanted from an unrelated, HLA-phenotypically identical and mixed lymphocyte culture (MLC)-nonreactive donor. Patients who received an allogeneic marrow graft were arbitrarily subdivided into two groups according to age (group 1, 45 to 50 years; group 2, 51 to 62 years of age). In addition, we analyzed the actuarial disease-free survival of 153 patients aged 30 to 44 as well as 138 patients aged 20 to 29 who received an allogeneic transplant from an HLA-matched sibling during the same period of time. To allow for a comparison with patients over 45 years of age, we divided these patients into those considered a "good risk" (AML in first remission, CML in chronic phase, preleukemia) and those considered at "high risk" (AML in relapse or second or subsequent remission, CML in accelerated phase or blast crisis) at the time of marrow grafting. Details of the transplantation procedure have been described elsewhere.[15] Patients with aplastic anemia were conditioned for grafting with cyclophosphamide (Cy), 50 mg/kg/d on each of four successive days. Patients with a hematologic malignancy were conditioned with a combination of Cy 60 mg/kg on each of two successive days followed by total body irradiation (TBI) delivered from dual ^{60}Co sources at an exposure rate of 5 to 8 cGy/min Allogeneic recipients received either methotrexate (MTX) or cyclosporine (CSP) for GVHD prophylaxis [16–18] Eight patients were given marrow that had been treated in vitro with monoclonal antibodies and complement.[19]

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Table 1. Patient Characteristics

	Allogeneic Transplantation		Syngeneic Transplantation
	Group 1	Group 2	
Number of patients	26	13	24
Age (years)*	47.5 (45-50)	55.5 (51-62)	53 2 (46-68)
Diagnosis (number of patients)			
AML, remission	8	1	5
AML, relapse	4	5	4
ALL, relapse	1	—	—
CML, chronic phase	4	1	3
CML, accelerated phase or blast crisis	8	2	1
Preleukemia	1	1	1
Non-Hodgkin's lymphoma	—	1	3
Aplastic anemia	—	2	2
Multiple myeloma	—	—	2
Hairy cell leukemia	—	—	1
Secondary leukemia	—	—	2

AML, acute myeloid leukemia, ALL, acute lymphoblastic leukemia, CML, chronic myeloid leukemia.
*Mean (range)

Acute and chronic GVHD were graded and treated as described.[20-23] The diagnosis of veno-occlusive disease (VOD) of the liver was made according to previously established criteria.[24] Diagnosis of interstitial pneumonia versus pneumonia due to bacterial or fungal infection was generally made by open-lung biopsy or at autopsy. Methods of assessment of engraftment have been described previously.[20] Marrow transplantation protocols and consent forms for these studies were approved by the Institutional Review Boards of the Fred Hutchinson Cancer Research Center or the University of Washington School of Medicine Data were evaluated as of February 1, 1985

RESULTS

Syngeneic transplants. Sixteen of 24 recipients of syngeneic grafts have died; the remaining 8 (33%) are surviving 476 to 2,412 days after grafting (Table 2) Actuarial disease-free survival was 20% at 7 years (Fig 1). Of the 8 survivors, 5 are well (Karnofsky score 100%) and 3 have suffered a ralapse of leukemia. Deaths were mainly due to relapse (6 patients) and idiopathic interstitial pneumonia (3 patients). Only one syngeneic recipient developed VOD of the liver, even though all but one (UPN 967) received at least 10 Gy TBI in combination with high-dose chemotherapy.

Allogeneic transplants. Actuarial survival of all 39 patients was 11% at 7 years (Fig 1).

In group 1 (45 to 50 years old), 8 of 26 patients (31%) are surviving, 1 in leukemic relapse, 4 with chronic GVHD, and 3 without complications (Karnofsky score 90% to 100%). Six patients (23%) died of cytomegalovirus (CMV) pneumonia, 7 (27%) from bacterial or fungal septicemia (Fig 2), and 9 from miscellaneous causes (Table 3). Five patients developed clinical evidence of VOD of the liver, but this was lethal in none. In group 2 (51 to 62 years old), 12 of 13 patients have died and 1 is alive 95 days after transplantation. Causes of death are listed in Table 4. Three patients had a leukemic relapse. Eight patients (62%) developed interstitial pneumonia (IP), due to CMV in 3 patients and of undefined etiology in 5 patients (Fig 2) All but one died. VOD of the liver was present in 3 patients but lethal in none. Of 9 patients with leukemia in this group, 7 were transplanted while in relapse or accelerated phase of the disease. With regard to other transplant-related problems, there was no difference between patients in groups 1 and 2.

Five of 12 "good risk" patients (41.5%) in groups 1 and 2 (AML in first remission, CML in chronic phase, preleukemia) are alive; 4 are well, 1 is alive but relapsed (UPN 2527), and 2 have chronic GVHD. Actuarial disease-free survival of these patients was 22% at 3 years (Fig 3). However, of 20 "high risk" patients transplanted (2 patients with aplastic anemia and 5 patients given HLA-incompatible transplants were excluded), only 2 (10%) became long-term survivors, with an actuarial disease-free survival at 3 years of 15%. Among 8 patients transplanted for AML in first remission, only 1 is alive and well, 1 is alive after relapse, and 6 have

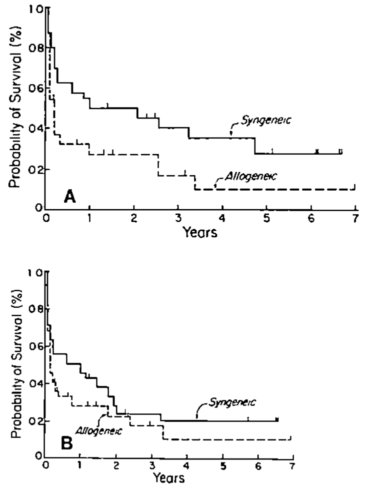

Fig 1. Kaplan-Meier product limit estimates of (A) overall survival and (B) disease-free survival in 24 syngeneic and 39 allogeneic transplant patients who were 45 years of age or older at the time of transplantation (HLA-partially compatible grafts are included).

Table 2. Outcome of Syngeneic Marrow Transplantation in Patients Aged 45 Years and Over

Unique Patient Number	Age (years)	Sex	Diagnosis*	Conditioning Regimen†	Outcome and Day‡	Cause of Death/Late Complications§
793	57	M	AML relapse	1	D 1,188	Adenocarcinoma of colon
858	48	M	HCL	1	A 2,412	—
862	46	F	AML first remission	1	A 2,408	CML 5 years later
871	50	M	Multiple myeloma	1	D 1,759	Recurrent myeloma
921	53	F	AML remission	2	D 63	Relapse
935	58	M	AML	1	D 98	Cerebrovascular insult
961	46	F	CML chronic phase	DMB + 1	A 2,167	CML, cytogenetic relapse
967	54	M	Aplastic anemia	4	A 2,161	—
1013	59	M	AML relapse	1	D 771	Relapse
1045	68	M	AML relapse	1	D 237	Relapse
1090	49	M	MDS	1	D 22	Found dead in bed
1143	51	F	NHL	1	A 1,807	Relapse
1170	46	F	AML following earlier chemoradiotherapy for Hodgkin's disease	1	D 385	Severe malnutrition, restrictive lung disease
1314	53	M	AML without prior therapy	2	D 50	Septicemia, acute respiratory distress syndrome
1565	49	F	AML first remission	2	D 330	Relapse
1648	47	M	CML accelerated phase	2	D 955	Relapse
1737	60	M	Multiple myeloma + AML	2	D 106	Idiopathic IP
1802	49	F	CML chronic phase	2	A 966	—
1823	46	M	AML first remission	2	A 770	Relapse
1918	61	M	NHL	2	D 31	Acute renal failure
2245	64	F	Aplastic anemia	4	A 476	—
2269	51	F	AML following earlier chemoradiotherapy for Hodgkin's disease	2	D 43	Idiopathic IP
2400	46	F	AML relapse	3	D 17	Idiopathic IP
2419	60	F	NHL	3	D 71	CMV IP

*AML, acute myeloid leukemia; CML, chronic myeloid leukemia; HCL, hairy cell leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma.

† *1*, cyclophosphamide (Cy) 60 mg/kg IV × 2 + 10 Gy single-dose total body irradiation (TBI); *2*, Cy × 2 + 6 × 2 Gy TBI over six days, *3*, Cy × 2 + 7 × 2 25 Gy TBI over seven days, *4*, Cy 50 mg/kg IV for four consecutive days, DMB, dimethylbusulfan 5 mg/kg IV

‡D, dead; A, alive as of February 1, 1985

§CMV, cytomegalovirus, IP, interstitial pneumonia

died of various causes. However, of 5 patients transplanted in chronic phase of CML, 3 are alive and well.

For the purpose of comparison, 153 patients aged 30 to 44 years and 138 patients aged 20 to 29 years were evaluated for survival. Figure 4 represents the Kaplan-Meier plots of disease-free survival for both "good risk" and "high risk" patients. At 3 years there were no differences among the "high risk" patients of all age groups. However, the younger patients (20 to 29 years) with a favorable diagnosis at transplant ("good risk") had a better survival rate than recipients older than 30 years: 48% for patients aged 20 to 29 years, 29% for patients aged 30 to 44 years, and 22% for those over 44 years. Overall long-term survival of patients aged 20 to 29 years and 30 to 44 years did not differ. Furthermore, survival curves of "high risk" patients of different age groups did not show any significant survival advantage for a particular age group after 3 years.

In terms of cardiac complications, two recipients developed atrial fibrillation, which was successfully treated. One patient (UPN 1090) presumably died from a cardiac arrhythmia. One patient (UPN 1821) suffered from cardiomegaly and pericardial effusion during conditioning therapy, but this problem resolved subsequently.

Regarding the actuarial incidence of acute GVHD, only patients surviving beyond day 21 after marrow grafting were evaluated. Recipients who died earlier were censored on the Kaplan-Meier plot. The actuarial probability of acute GVHD of grades II through IV was 30% in group 1 and 79% in group 2. This difference was statistically significant ($P < .05$, Fisher's exact test). Furthermore, the onset of acute GVHD was earlier in group 2 than in group 1 ($P < .05$,

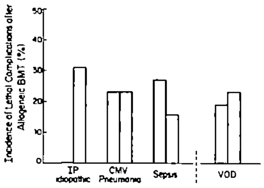

Fig 2. Schematic presentation of the incidence of lethal complications (idiopathic interstitial pneumonia, CMV pneumonia, septicemia) in allogeneic graft recipients between 45 and 50 years of age (group 1) compared to those 51 to 62 years of age (group 2). Shown also is the frequency of VOD, although this complication was not the immediate cause of death in any patient. ▨, Age 45–50 years; □, Age >50 years.

Table 3. Outcome of Allogeneic Marrow Transplantation in Patients 45 to 50 Years of Age (Group 1)

Unique Patient Number	Age (years)	Sex	Diagnosis*	Conditioning Regimen†	GVHD Prophylaxis‡	Acute GVHD (grade)	Chronic GVHD	Outcome and Day§	Cause of Death/Late Complications‖
798	47	M	AML first remission	Cy × 2 9 2 Gy	MTX	III	—¶	D 86	CMV IP
800	50	F	CML blast crisis	2	MTX	II	yes	A 2,524	—
830**	49	M	ALL relapse	2	MTX	I	—	D 50	Septicemia (*Candida*)
867	46	M	CML blast crisis	2	MTX	—¶	—	D 13	Septicemia (*Corynebacterium*)
1153	45	M	AML first remission	1	MTX	0	—	D 35	Aspiration pneumonia/VOD
1593	46	M	AML relapse	3	MoAB, MTX	III	—	D 52	*Aspergillus* abscesses in brain and CMV IP/VOD
1595	46	M	AML first remission	2	MTX	0	no	A 1,100	—
1641	46	M	AML first remission	2	MTX, CSP	II	—	D 40	CMV IP
1646	47	M	CML chronic phase	2	MTX	I	yes	D 924	Relapse
1757	48	F	AML relapse	3	CSP	III	—	D 59	Relapse
1801	45	F	AML second remission	1	MTX	0	yes	A 855	—
1820	47	M	AML relapse	3	MTX	—	—	D 11	Septicemia
1821	45	F	CML accelerated phase	Cy × 2 4 × 2 25 Gy	MTX	—	—	D 5	Cardiomegaly, renal failure after marrow infusion
1843	49	F	AML first remission	2	CSP	I	yes	D 137	Septicemia (*Pseudomonas*)
1978	45	M	AML relapse	3	MoAb, MTX	—	—	D 53	Septicemia (no engraftment)
2043	45	M	CML blast crisis	3	MTX	0	—	D 31	Septicemia (*Candida*)/VOD
2069	50	M	CML accelerated phase	3	CSP	I	yes	D 352	Relapse
2115	47	M	CML accelerated phase	3	MoAb, CSP	0	—	D 65	CMV IP
2123	48	M	CML chronic phase	2	MoAb, CSP	0	no	A 521	—
2196	45	M	CML chronic phase	2	CSP	0	yes	A 469	—
2316	47	M	CML accelerated phase	3	CSP	0	—	D 38	CMV IP/VOD, atrial fibrillation
2380	48	M	AML first remission	2	MTX, CSP	II	—	D 77	CMV IP
2439**	49	M	CML chronic phase	2	MTX	I	yes	A 246	—
2527	47	M	AML first remission	2	MTX, CSP	0	no	A 169	Relapse
2635	47	F	Preleukemia	2	MTX, CSP	0	—	A 65	—
2643	50	M	CML blast crisis	3	MoAb, MTX	0	—	D 21	Septicemia (*Candida*)/VOD

*ALL, acute lymphoblastic leukemia, AML, acute myeloid leukemia, CML, chronic myeloid leukemia

† *1*, cyclophosphamide (Cy) 60 mg/kg IV × 2 + 10 Gy single-dose total body irradiation (TBI), *2*, Cy × 2 + 6 × 2 Gy TBI over six days, *3*, Cy × 2 + 7 × 2 25 Gy TBI over seven days

‡CSP, cyclosporine after grafting, MoAb, in vitro treatment of donor marrow with monoclonal antibodies and complement, MTX, methotrexate after grafting

§D, dead, A, alive as of February 1, 1985

‖CMV IP, cytomegalovirus interstitial pneumonia, VOD, veno-occlusive disease of the liver

¶Patient died too early to be evaluable

**Donor differed from recipient for one HLA antigen

Mantel-Cox test). Of 15 long-term survivors (≥day 95) in groups 1 and 2, 10 developed chronic GVHD.

DISCUSSION

Marrow transplantation offers the chance for long-term survival and possibly cure for patients with a hematologic malignancy or aplastic anemia. However, applicability may be limited by several factors including patient age. Numerous studies have shown an increase of transplantation-related complications in older patients,[8–11] and most centers will not accept patients over 40 or 45 years of age for transplantation. It is not clear, however, whether it is judicious to exclude all patients from marrow grafting because of an arbitrarily chosen age limit.

In the present retrospective review, we analyzed our results with syngeneic and allogeneic bone marrow transplantation in 63 consecutive patients 45 to 68 years of age. Some encouraging results and several problems were noted. The frequency of pulmonary complications presumably related to chemoradiotherapy was high in allogeneic recipients of more than 50 years of age. Eight of 13 recipients (62%) developed pulmonary complications, and 7 died of respiratory failure. The incidence of interstitial pneumonia in patients between 45 and 50 years of age (23%) was not different from the incidence in younger age groups.[25,26] One could speculate that the lungs of older patients are increasingly susceptible to respiratory complications caused by aggressive conditioning regimens. However, nearly all patients over 50 years of age were transplanted in an advanced stage of leukemia and had received multiple cycles of pretransplant chemotherapy, circumstances under which interstitial pneumonia appears to be more common regardless of age.[11,26] It is also of note that of 8 patients transplanted in first remission of AML, only 1 is alive and well, in contrast to 3 of 5 recipients grafted for CML in chronic phase. This might point once more to the negative impact of previous chemotherapy, particularly in elderly patients, since patients with CML in chronic phase usually receive less intensive treatment prior to marrow grafting.

The observation that "high risk" patients over 44 years of age had a survival rate comparable to that of recipients 30 to 44 years of age may be important when counseling older patients. A comparison between "good risk" patients beyond 3 years after grafting is not possible, since the longest survivor over 44 years of age is only 3 years after transplantation. At 3 years, however, actuarial disease-free survival is not statistically different. These data suggest that a negative effect of age on prognosis after marrow transplantation becomes prominent already around age 30. Thus survival of patients transplanted when 45 years of age or older, although inferior to that in patients less than 30 years of age, may not

Table 4. Outcome of Allogeneic Marrow Transplantation in Patients Over 50 Years of Age (Group 2)

Unique Patient Number	Age (years)	Sex	Diagnosis*	Conditioning Regimen†	GVHD Prophylaxis‡	Acute GVHD (grade)	Chronic GVHD	Outcome and Day§	Cause of Death/Late Complications‖
848	53	M	CML accelerated phase	2	MTX	III	—	D 58	CMV IP
979	61	M	Aplastic anemia	4	MTX	—	—	D 23	Idiopathic IP
984	53	M	Aplastic anemia	4	MTX	IV	—	D 65	CMV IP
998	52	F	AML relapse	2	MTX	III	yes	D 1,225	Gastrointestinal bleeding
1422	62	M	NHL	2	MoAb	II	yes	D 115	Relapse, toxoplasma encephalitis
1430	53	M	AML first remission	2	MoAb, MTX	0	—	D 37	Gastrointestinal bleeding (stress ulcer)/VOD
1577	55	F	AML relapse	3	MoAb, MTX	I	no	D 887	Interstitial pulmonary fibrosis
1636¶	54	M	Preleukemia	1	MTX, CSP	III	yes	D 128	Idiopathic IP, relapse
1699	53	F	AML relapse	2	MoAb, MTX	III	—	D 58	CMV IP
1732	54	M	AML relapse	3	MoAb	—	—	D 18	Septicemia (*Klebsiella, Candida*)
1994	58	M	AML relapse	2	MTX	0	—	D 65	Idiopathic IP/VOD, relapse
2588**	51	F	CML chronic phase	1	MTX	II	—	D 32	Septicemia (*Candida*), VOD
2594¶	58	M	CML accelerated phase	3	MTX	III	no	A 95	Idiopathic IP

*AML, acute myeloid leukemia, CML, chronic myeloid leukemia, NHL, non-Hodgkin's lymphoma

† *1*, cyclophosphamide (Cy) 60 mg/kg IV × 2 + 10 Gy single-dose total body irradiation (TBI), *2*, Cy × 2 + 2 Gy TBI over six days, *3*, Cy × 2 + 2 25 Gy over seven days; *4*, Cy 50 mg/kg IV for four consecutive days.

‡CSP, cyclosporine after grafting; MoAb, in vitro treatment of donor marrow with monoclonal antibodies and complement; MTX, methotrexate after grafting.

§D, dead; A, alive as of February 1, 1985.

‖CVM, cytomegalovirus, IP, interstitial pneumonia, VOD, veno-occlusive disease of the liver

¶Donor differed from recipient for two HLA antigens

**Transplanted from an HLA-phenotypically unrelated donor.

differ significantly from that in patients of the intermediate age group (30 to 44 years old).

Another concern in older patients was the development of hepatic VOD, since a previous study had shown that the risk of VOD increased with patient age.[24] In the present population of older patients, however, the incidence of VOD was not increased in comparison to younger patients, and, in fact, only 1 of 24 recipients of a syngeneic transplant developed VOD. Since VOD is not thought to be mediated by an immunologic process, this low incidence is surprising. However, the small numbers of patients do not allow for a more detailed statistical analysis.

The frequency of infectious complications early after transplantation was high. One possible explanation for this finding might be a reduced thymus function. Thymus involution begins at about 20 years of age, and circulating levels of thymic hormones are decreased;[27] this could result in a defective maturation of lymphoid cells derived from the transplanted marrow.

The actuarial incidence of acute GVHD for patients 45 to

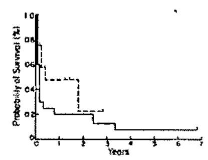

Fig 3 Kaplan-Meier product-limit estimates of disease-free survival in 12 patients aged 45 years and older who received an HLA-identical allogeneic transplant for AML in first remission, CML in chronic phase, or preleukemia ("good risk"; dashed line) compared to those patients (n – 20) who were at "high risk" at transplant (acute leukemia in relapse or second or later remission, CML in accelerated phase or blast crisis; solid line). Tick marks indicate surviving patients.

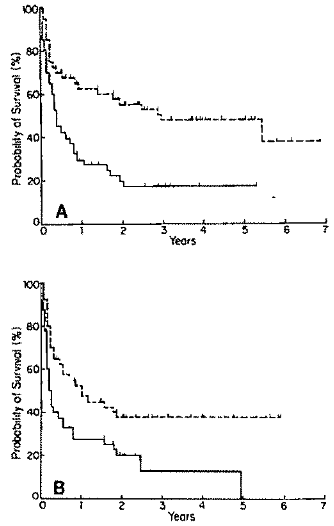

Fig 4. Kaplan-Meier product-limit estimates of disease-free survival in patients between ages 20 and 29 (A) and ages 30 and 44, (B), categorized by recipient risk at transplant ("good risk," dashed line; "high risk," solid line; see Fig 3) between January 1, 1978, and December 1, 1984.

50 years of age was similar to that observed in recent studies in younger patients[5,16,18,22] In patients over 50 years of age, however, the incidence of acute GVHD was high (79.5% grades II through IV). Because of the small number of patients and the fact that three had received partially matched transplants, these data should be interpreted cautiously. Somewhat surprisingly, chronic GVHD was neither more frequent nor more severe than in younger patients.[22]

Although this retrospective analysis deals with a heterogenous patient population (including T cell–depleted marrow transplantation and HLA–partially matched grafts), some preliminary conclusions can be drawn from these data. Marrow transplantation can be carried out successfully in patients aged 45 and over, albeit with a higher morbidity, especially with regard to interstitial pneumonia, acute GVHD, and various infectious complications. As in other age groups, recipients of syngeneic transplants did somewhat better than recipients of allogeneic transplants, but relapse of leukemia was still a significant problem.

Problems were apparent particularly in patients over 50 years of age, possibly because of a bias toward poorer-risk patients in this group. Therefore, patients with leukemia who are over 45 years of age and have an identical-twin donor are definitely candidates for marrow transplantation. With an allogeneic donor, selected patients may be considered for marrow grafting: Patients with CML in chronic phase seem to benefit from allogenic marrow grafting, as do patients with preleukemia. However, patients with acute leukemia at the present time should be considered for transplantation only at first relapse rather than in first remission.

The number of patients with aplastic anemia was too small to draw any final conclusions. Since immunosuppressive therapy has been reported to have a 40% to 60% response rate,[28,29] it might be reasonable to try this treatment first before proceeding to allogeneic marrow grafting.

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Short-Term Culture of Acute Myeloid Leukemia Blasts: Analysis of Acquired Susceptibility to Activated Natural Killer Cells

By Philippe Moingeon, Arnaud Ythier, Alexandre Nowill, Laurence Delmon, Chantal Bayle, José-Luis Pico, Claude Bohuon, and Thierry Hercend

Following a cryopreservation step, short-term cultures of circulating leukemic blasts from a patient with acute myeloid leukemia (AML) were performed. Because cultured tumor cells became susceptible to natural killer (NK) activity, in vitro alteration of the blasts was studied. Immediately after thawing, cell suspensions consisted of a relatively homogeneous population of undifferentiated blasts. In culture, tritiated thymidine uptake by the leukemic cells was low during the first 24 hours and then increased (×20) to a peak on day 7. The cell concentration started to increase on day 4. On day 8, <10% of the cultured cells still appeared as undifferentiated blasts, whereas up to 60% were granular and 30% to 40% had a monocytoid morphology. Prior to being cultured, the blasts were resistant to resting and IL2-activated natural killing. When the kinetics of in vitro acquired susceptibility were studied, it was found that maximum cytotoxicity against these leukemic cells was reached within 24 hours. Thus, the blasts had become NK-sensitive prior to increase in DNA synthesis, proliferation, and differentiation based on morphological and cytochemical criteria. In contrast, there was a positive correlation between acquired susceptibility and surface expression of an activation antigen, termed TNKtar. To dissect further the mechanisms of acquired susceptibility, a series of six NK clones representing four distinct phenotypes of NK active lymphocytes were tested against the leukemic cells. Immediately after thawing, blasts were essentially resistant to all clones, whereas they were strongly killed by 5 of 6 clones when cultured for 24 hours. Cold target inhibition assays indicated that resistance of fresh blasts was likely to be due to a binding defect. These results suggested that tumor cells became susceptible because they surface-expressed NK target structure(s) in the early phase of an activation process leading to their proliferation and/or differentiation. This hypothesis was substantiated for one clone, termed JT9, because the anti-TNKtar antibody blocked cytotoxicity of JT9 cells against the cultured blasts.

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USING long-term cultured hematopoietic cell lines such as K562, a series of studies have been performed to correlate stages of target cell differentiation and susceptibility to natural killer (NK) activity.[1–3] Various agents, including sodium butyrate and phorbol esters, were used to obtain further differentiated and well-characterized phenotypes of K562 cells, which were then tested as target cells for human peripheral blood NK active lymphocytes. When cytotoxicity against either untreated or differentiated K562 cells was compared, it was found that the latter were less sensitive. Because for example, certain highly differentiated tumors (ie, myelomas) are quite susceptible to natural killing, it has been pointed out that results obtained with K562 cells should not be interpreted in a simplistic fashion.[1] However, these experiments demonstrated that cellular events leading to varying differentiation stages of a particular cell type are important in determining susceptibility to NK cells. Moreover, they pointed out that in vitro experimental systems in which cell alteration is carefully monitored may be of great interest in dissecting the mechanisms by which NK active lymphocytes can recognize and kill tumor cells.

We analyzed a situation in which undifferentiated fresh leukemic blasts resistant to NK cells became susceptible following a short-term culture. A series of biological parameters was investigated for an eight-day period. A large fraction of cultured leukemic blasts underwent an activation process leading to proliferation and/or differentiation. However, NK susceptibility was acquired very early (within 24 hours of culture), prior to both increased DNA synthesis and detectable morphological changes. Experiments performed with well-characterized NK clones strongly suggested that early expression of unique surface antigens, in the context of tumor cell activation, represented a necessary signal for target recognition and subsequent lysis.

MATERIAL AND METHODS

Case report A 19-year-old woman was admitted to the Hematology Unit of Institut Gustave-Roussy for acute myeloblastic leukemia, type M1, according to French-American-British (FAB) classification. The initial WBC count was 104,000/μL Between April and November 1984, the patient received two successive courses of daunorubicin, vincristine, and Ara-C, two courses of Ara-C plus *m*-AMSA, two courses of etoposide, *m*-AMSA, and melphalan and, finally, one cycle of therapy combining aclacynomycine and 6-thioguanine Because complete remission was not achieved, we decided to administer symptomatic treatment.

In vitro culture of leukemic cells Prior to therapy, 2×10^9 leukemic cells were obtained following Ficoll-Hypaque density gradient centrifugation. These cells were immediately cryopreserved (50 vials containing 40×10^6 cells) in the presence of a final concentration of 10% dimethyl sulfoxide (DMSO). Cells were thawed in RPMI containing 2.5% human AB serum and then seeded at 3×10^5 cells per milliliter in culture medium containing 15% human AB serum, 1% penicillin-streptomycin; 1% sodium pyruvate, and 2% L-glutamine In this article, cells studied immediately after thawing are termed "fresh" or "day 0" blasts. For morphological and cytochemical studies, cytocentrifuge smears were stained with

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May-Grunwald-Giemsa, Sudan black B, and α naphtyl-butyrate esterase.

Thymidine incorporation and cell cycle analysis. For thymidine incorporation assays, cells were seeded at 50,000 cells per well in round-bottomed microtiter plates Experiments were done in culture medium. Cultures were pulsed as described in the Results section with 0 2 μCI of tritiated thymidine and, subsequently harvested on a MASH II apparatus Tritiated thymidine incorporation was measured using a liquid scintillation counter For cell cycle analysis 1 × 10^6 day 0, day 1, and day 2 leukemia cells, and 1 × 10^6 peripheral blood mononuclear cells (PBMCs) from a normal donor were treated in parallel They were fixed with 1% formaldehyde in phosphate-buffered saline (PBS). Ethidium bromide (Calbiochem, San Diego, Calif) was added, to a concentration of 80 μg/mL. After ten minutes of equilibration at room temperature, cells were analyzed on a Becton Dickinson FACS IV. Intensity of fluorescence was determined by analyzing 10,000 cells per sample.

Immunofluorescence assays and cytofluorometric analysis. Phenotypic analysis was performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse immunoglobulin (Coulter Immunology, Hialeah, Fla), as previously described.[4] Samples were analyzed either on an Epics V flow cytometer (Coulter Electronics) or a FACS 440 (Becton Dickinson). Each sample contained 10,000 cells. Histograms display the number of cells (ordinate) *v* the intensity of fluorescence (abscissa). The negative control used to determine background fluorescence was an ascites containing an antibody previously shown not to react with the tested cells (ie, anti-B1 for NK clones)

Cytotoxicity assays. Cytotoxicity assays were performed in four hours against chromium-labeled target cells. All experiments were done in culture medium using V-bottomed microtiter plates according to a standard method Percentage of specific cytotoxicity was calculated as previously described.[4] Purified recombinant IL-2 (Biogen) used to activate resting NK cells from normal donors was used at a final concentration of 70 ng/mL.

Uncloned effector cells. PBMCs from healthy donors were separated by Ficoll-Hypaque density-gradient centrifugation Cell suspensions enriched in NK activity were obtained from PBMCs by centrifugation on discontinuous density gradients of Percoll after a step of adherence on nylon wool columns.[5] Gradients consisted of six layers ranging from 47% to 31% Percoll. Under our experimental conditions, fraction 3 consistently contained >65% large granular lymphocytes and was used for the cytotoxicity assays shown in Table 1. Fraction 5 consistently contained >90% small lymphocytes and was used as a negative control.

Human NK clones Methods for generation of human NK cloned cell lines have been described in detail.[4,6,7] In brief, for initial cloning cells are usually seeded at 1 or 0.5 cells per well on a feeder layer of autologous or allogeneic irradiated (5,000 rad) PBMCs plus PHA (2 μg/mL) and/or allogeneic irradiated (5,000 rad) Epstein-Barr virus (EBV)-transformed B cells Colonies are expanded by addition of culture medium containing leukocyte-conditioned medium (LCM) (10%–15% final dilution) every three days LCM is produced following stimulation of whole PBMCs at a concentration of 2.5 × 10^6 cells per milliliter by a combination of phytohemagglutinin (PHA), phorbol myristate acetate (PMA), and irradiated EBV-transformed B cells as previously described.[4] Cells are washed four times after three hours to remove the mitogens, and supernatants are harvested after 40 hours of incubation at 37 °C.

All clones described in these studies have been subcloned at least three times at 100 cells per well. They all have homogeneous phenotypes when tested with a series of monoclonal antibodies. Phenotypes are stable and have been controlled after each subcloning procedure. Of the clones used in these studies, JT3 and JT9 have been previously described in detail.[6,7] TC12, TC48, 50, and 59 have been derived from peripheral blood lymphocytes of a leukemic patient during early reconstitution following allogeneic bone marrow transplantation Cell surface phenotypes have been determined using indirect immunofluorescent assays and subsequent cytofluorometric analysis. Anti-T3 defines all mature T lymphocytes in human peripheral blood [8] T4 and T8 antigens are expressed on reciprocal subpopulations of circulating T lymphocytes.[9,10] Anti-T11 defines an antigen associated with the sheep erythrocyte receptor.[11] NKH1A is a pan NK-associated marker, whereas NKH2 is expressed on a subpopulation of circulating LGL and a fraction of human NK clones [12] The reactivity of the clones with these six monoclonal antibodies (Mabs) is shown in Table 1A. All Mabs reacted with either <5% or >90% of cloned cells in each case As shown in Table 1, TC48 represents an uncommon lymphocyte reacting only with anti-NKH1A. JT9 cells[7] have a mature T cell phenotype coexpressing T3, T8, and T11. However, as opposed to conventional T lymphocytes, JT9 cells are NKH1A+. JT3 and TC59 have usual NK phenotypes, both being T3−, T11+, NKH1A+, NKH2+. TC12 and T50 are also T3−, T11+, and NKH1A+ but, as opposed to JT3 or TC59, these clones do not express NKH2. Functionally, these six clones are strongly cytotoxic against K562 and JM (Table 1B), used here as conventional, highly susceptible NK target cell lines For optimal standardization, experiments were repeatedly performed with identical aliquots of thawed cloned effector cells.

Table 1.

A. Surface Phenotype of Cloned NK Cell Lines

Clone	T3[*]	T4	T8	T11	NKH1A	NKH2
JT3	−[†]	−	−	+	+	+
JT9	+	−	+	+	+	−
TC12	−	−	−	+	+	−
TC48	−	−	−	−	+	−
TC50	−	−	−	+	+	−
TC59	−	−	−	+	+	+

B Cytotoxic Activity of Clones Against Conventional Hematopoietic NK Target Cell Lines

Target Cell	JT3[‡]	JT9	TC12	TC48	TC50	TC59
K562	62[§]	62	76	58	68	77
JM	74	64	70	65	65	75

[*] Expression of antigens determined by indirect immunofluorescence reactivity with specific monoclonal antibodies.

[†] − < 5% of cells reactive, + > 90% of cells reactive

[‡] E/T ratio 6.1.

[§] Mean of triplicate cultures, SD ≤5%.

RESULTS

Morphological and cytochemical analysis of fresh and in vitro cultured leukemia cells. Fresh peripheral blood leukemic blasts were cultured after a cryopreservation step and subsequent thawing First, it was macroscopically evident that at least a fraction of the cells started to divide within a few days. This either spontaneous or potentially DMSO-induced in vitro alteration was studied with a series of biological parameters, including conventional morphological and cytochemical analysis. Cytocentrifuge smears were prepared immediately after thawing (day 0) and again after 1, 2, 4, 6, and 8 days of culture. During this period, cell viability was always >95%.

On day 0, >95% of cytocentrifuged cells were undifferentiated blasts. This leukemic population appeared relatively homogeneous (maximal size was two times greater than that of a small resting lymphocyte) with a high nuclear-cytoplasmic (NC) ratio (Table 2). Cells did not contain granules and were not stained by Sudan black B or naphtyl butyrate esterase. No mitoses were seen in 10,000 cells examined. After 24 hours of culture, there were no major morphological changes. However, a slight decrease in N/C ratio and the emergence of granules in 10% to 15% of the cells was noted. Between day 1 and day 2 there was only a slight increase in the size of a fraction of the cells. Morphological transformation became evident on day 4 when 20% to 25% of the cells were granular. In addition, ~10% to 15% had a monocytoid aspect. On a representative smear, 23 mitoses per 1,000 cells were seen, suggesting a high degree of proliferation. After eight days of culture as much as 60% of the cells were granular and 30% to 40% were monocytoid. Less than 10% of cells had an undifferentiated blastic aspect; these cells were larger than their day 0 counterparts. The most differentiated myeloid cells appeared as metamyelocytes, although their morphology was abnormal because of an unusually large size and a maturation asynchronism between nucleus and cytoplasm. The onset of cytochemical markers was delayed and incomplete, suggesting enzymatic defects in the leukemic cells. In summary, morphological analysis indicated that a large fraction of the tumor cells underwent an in vitro activation process leading to proliferation and/or differentiation.

Thymidine incorporation and cell cycle analysis. Morphological analysis suggested that a fraction of the tumor cell entered S phase and subsequently proliferated. Thymidine incorporation was therefore investigated to analyze the kinetics of proliferation. Blast cells thawed on day 0 were cultured in triplicate. The first triplicate was pulsed immediately after thawing and harvested following 24 hours of culture. At that time, the second triplicate was pulsed and was harvested the next day. This procedure was repeated from day 0 to day 7. Prior to harvest, cultured cells were counted to determine cell concentration in the wells. Results of a representative experiment are shown in Fig. 1. Between day 0 and day 1, thymidine incorporation was at a background level of ~2,500 cpm. Subsequently, thymidine uptake rose progressively to peak on day 7 at ~50,000 cpm. Cell concentration in the cultures was stable for four days and then peaked on day 7 at 8×10^5/mL, whereas the blasts were initially seeded at 3×10^5/mL on day 0, indicating that a fraction of the cultured cells had strong proliferative capacity. During the first 24 hours of culture, thymidine uptake was very low, suggesting that most of the fresh leukemic blasts occurred in the GO/G1 phase of the cell cycle.

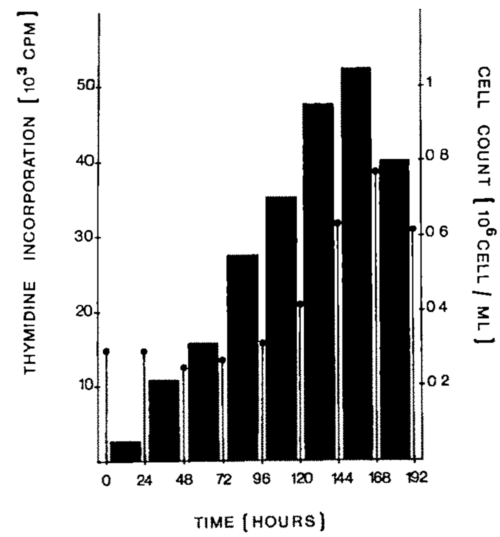

Fig 1. Tritiated thymidine uptake and cell concentration in leukemic blast cultures. (▮) Tritiated thymidine incorporation between time indicated on the left and time indicated on the right. For example, the first bar on left indicates thymidine uptake during first 24 hours of culture. (●) Cell concentration in culture at time indicated below.

To confirm this latter point, cells were treated immediately after thawing with ethidium bromide, which binds to double-stranded RNA as well as to DNA. Use of this reagent makes it possible to delineate the successive phases of the cell cycle from the resting GO stage (low RNA content and 2C DNA), through G1 (increased RNA content) and S/G2/M (DNA synthesis with >2C DNA content). Ethidium bromide staining suggested that most peripheral blood fresh blasts were in GO phase since their RNA and DNA content was not higher that that of control resting peripheral blood lymphocytes from a normal individual. Moreover, there was only a minimal increase ($\leq$5%) in fluorescent blast cells after 24 hours of culture. In contrast, after two days, ~20% of the cells were stongly fluorescent which, together with the

Table 2. Morphological and Cytochemical Analysis of Fresh and In Vitro Cultured Leukemia Cells

	DO*	D1	D2	D4	D6	D8
Cell size	1 to 2†	1 to 3	2 to 5	3 to 5	2 to 5	2 to 5
Basophilia	+/−	+	+	++	+	+
N/C ratio	75–90‡	60–80	50–80	50–70	40–80	40–70
Granular cells	<5‡	10–15	10–15	20–25	40–45	50–60
Monocytic cells	0	<5‡	<5	10–15	30–40	30–40
Sudan black B	–	<5‡	<5	<5	<5	15–20
Naphtyl-butyrate esterase	–	<5‡	<5	<5	<5	30
Mitosis	0§	1	5	23	12	17

*Day 0 means immediately after thawing; DAY 1· 24 hours of culture.

†1 to 2 times the size of a small resting lymphocytes

‡Percentage

§Number of mitoses per thousand, 10,000 cells were examined in each slide increased thymidine uptake, indicated a progression of some blasts through the G1/S/G2 phases of the cell cycle (not shown).

NK activity of allogeneic PBMCs against fresh and cultured leukemic cells. The NK activity of PBMCs and of large granular lymphocytes (LGL) (enriched or deprived fractions) was tested against fresh and cultured leukemia cells Effector cells were used either unstimulated or after an 18-hour treatment with recombinant IL 2. They were washed two times before being tested against chromium-labeled leukemia cells in a conventional four-hour assay. To standardize the experiment, blasts were harvested from bulk cultures on days 1 (24 hours of culture), 2, 4, 6, and 8 and were immediately frozen. Cryopreserved cells were then thawed simultaneously to perform the assays. Control experiments indicated that cryopreservation of the blasts at various times did not alter susceptibility to NK mediated lysis (data not shown).

Table 3 shows a representative experiment performed using peripheral blood from three normal donors known to have a relatively strong spontaneous lytic activity towards conventional NK target cell lines such as K562 or JM. Cytotoxicity of high-density lymphocytes (eg, small resting lymphocytes) is not presented in Table 3 because it was always at background levels (≤4%), even after treatment with IL2 PBMC and LGL-enriched fractions from all donors had no measurable activity against D0 (uncultured) leukemia cells. After rIL2 treatment, minimal cytotoxicity (6%) was observed only with LGL fraction from donor 3. In contrast, IL2 activated peripheral blood cells from the three donors displayed significant cytotoxicity against the cultured blasts. As expected, the strongest lytic activities were found in the LGL-enriched fractions which are known to contain the great majority of NK effector cells.[13] NK susceptibility was acquired very early during the course of the leukemia cell cultures as evidenced by the high degree of cytoxicity measured against D1 chromium-labeled blasts (≤34 percent specific cytotoxicity in donor 3). Subsequently decreasing but maintained cytoxicity was measured against D2, D4, and D6 leukemic cells.

Table 3. Resting and IL2 Activated Natural Killing Against Fresh and Cultured Leukemia Cells

			Leukemia Cells				
			D0	D1	D2	D4	D6
Donor I*	PBMC	Medium†	0	1	3	0	0
		IL2	0	4	3	1	2
	LGL‡	Medium	0	5	4	1	0
		IL2	0	23	11	7	9
Donor II	PBMC	Medium	0	2	2	1	0
		IL2	0	10	6	4	3
	LGL	Medium	0	2	3	1	0
		IL2	0	15	8	4	6
Donor III	PBMC	Medium	0	3	3	1	2
		IL2	0	14	15	11	8
	LGL	Medium	0	7	8	5	1
		IL2	6	34	29	21	21

*E/T ratio 40.1, percent specific cytotoxicity, mean of triplicate cultures, SD ≤5%

†Cells were incubated for 18 hours with either medium or recombinant IL2 (final concentration of 70 ng/ml in culture medium) for 18 hours prior to addition of chromium labeled target cells

‡Low density cells enriched in large granular lymphocytes (see Materials and Methods section)

Cytotoxicity of NK clones against fresh and cultured leukemic cells. The above experiments suggested that in vitro alteration of leukemic blasts was accompanied by an increased susceptibility to NK activity, particularly following IL2 treatment of effector cells. To study this phenomenon further, a series of human NK clones were used as effector cells in the same experimental system. These NK-cloned cell lines are monoclonal populations of IL2-dependent activated NK cells, which have been initially selected because of their non-MHC–restricted lytic capacity toward a series of tumor cells, including K562.

Table 4 shows that the NK clones used here either did not kill uncultured blasts (JT3, JT9, TRC48, or 49) or displayed a low level of cytotoxicity against these cells (TRC12, 50). However, each cloned cell line except JT3 was strongly lytic against the cultured blasts. Note that under these experimental conditions, cytotoxicity of all clones, including JT3, was >60% against K562 cells (Table 1B). Correlating results obtained with IL2 stimulated peripheral blood cells, maximum cytotoxicity displayed by the clones was reached as early as day 1. For example, using TC12 as effector cells, specific cytotoxicity rose from 8% on day 0 to 54% on day 1. Subsequently, decreasing but maintained cytotoxicity was observed against blasts cultured for 2, 4, 6, and 8 days.

Cold target inhibition experiments. To investigate why resistant fresh blasts became susceptible to most NK cells tested following a short-term culture, cold target inhibition experiments were performed. Cytotoxicity of TC12 and TC50 cells was tested against chromium-labeled D2 blasts in the presence of medium or increasing numbers of either cold D0 blasts, cold D2 blasts or cold K562 cells. Results shown in Fig 2 were similar for clones TC12 and TC50, which both displayed low levels of cytotoxicity against fresh blasts (Table 4). It was found that D0 leukemic cells were poor cold target competitors; at a 20/1 cold/hot target ratio, inhibition was 28% and 22% using TC12 and TC50 cloned effector cells, respectively. In contrast addition of either cold D2 blasts or K562 cells induced a strong inhibition of measured cytotoxicity. For example, at 20/L cold/hot target ratio, using TC12 as effector cells, 72% and 88% inhibition were obtained with D2 blasts and K562 cells, respectively. These

Table 4. Cytotoxicity of NK Clones Against Fresh and Cultured Leukemia Cells

	Days					
Clone	0	1	2	4	6	8
JT3*	0†	0	4	4	3	0
JT9	0	30	29	28	22	17
TC12	8	54	34	30	26	21
TC48	0	35	17	25	19	19
TC50	7	35	13	20	17	14
TC59	0	51	40	36	21	14

*E/T ratio 10:1

†Mean of triplicate cultures, SD ≤5%.

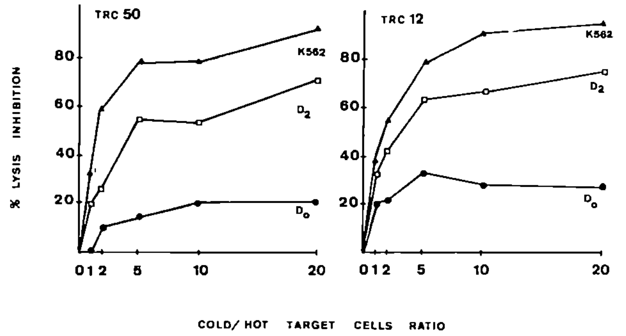

Fig 2. Cold target inhibition assays. Cytotoxicity was measured following four-hour assays. First TC50 and TC12 cloned cells were plated. Subsequently, either cold D0 blasts, D2 blasts, or K562 cells were seeded at a cold/hot ratio as indicated. Finally, radiolabeled D2 blasts were added at 5,000 cells per well. Experiments were performed in triplicate cultures. SD for each point was <12%. Effector/radiolabeled target ratio was 5:1.

results suggested a binding defect between cloned NK cells and fresh leukemic blasts.

Receptors involved in cytotoxic reactions against leukemic blasts. In previous studies, we demonstrated that a surface structure expressed on lymphoid cells within 24 hours following mitogen activation is likely to represent a target antigen for a series of NKTa+ cloned NK cell lines, including JT9.[7,14] It was of interest to investigate the expression of this molecule, termed TNKtar, on the membrane of either fresh or short-term-cultured leukemic blasts. Indirect immunofluorescence assays were performed and followed by cytofluorometric analysis It was found (Fig 3) that TNKtar was either expressed with very low density or was absent on D0 blasts; however, after 24 hours of culture, anti-TNKtar strongly reacted with most of the leukemic cells. This expression was then maintained during the eight days of culture (Fig 3) Because TNKtar surface expression correlated with acquired susceptibility to NK clones including JT9, blocking experiments were conducted. The cytotoxicity of JT9 cells was tested against D0, 1, 2, 4, and 6 blasts. JT9 cells and leukemic blasts were treated with anti-NKTa (used here as a positive control) and anti-TNKtar,[14] respectively. NKTa and TNKtar have been previously shown to recognize at the effector[7] and target cell level[14] receptors involved in the cytotoxic reactions mediated by JT9 against conventional NK target hematopoietic cell lines. JT9 cells were incubated with either medium or a saturating concentration of anti-NKTa for three hours at 37 °C. Subsequently, chromium-labeled blasts were added. Reciprocally labeled leukemic cells were incubated with saturating concentration of anti-TNKtar for one hour at 37 °C prior to addition of JT9 cells. As shown in Table 5, it was found that anti-NKTa and anti-TNKtar were both able to block these cytotoxic reactions significantly. For example, against D1 blasts, inhibition of lysis induced by anti-NKTa was 66%; anti-TNKtar treatment resulted in a 56% inhibition.

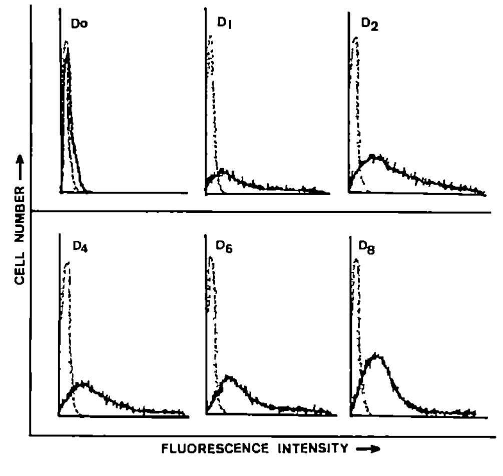

Fig 3. Surface expression of TNKtar antigen during in vitro cultures. Surface expression of TNKtar on leukemic cells was measured using indirect immunofluorescence assays followed by cytofluorometric analysis on a FACS 440. The diagrams represent number of cells (ordinate) v intensity of fluorescence (abscissa) expressed on an arithmetic scale; (· · ·) background fluorescence obtained with the unreactive anticlonotypic NKTa monoclonal antibody; (——) reactivity of anti-TNKtar. The percentage of TNKtar+ cells in the culture was evaluated as follows: day 0, 4%; day 1, 68%; day 2, 66%; day 4, 75%; day 6, 70%; day 7, 56%.

JM, a long-term-cultured cell line derived from a T cell leukemia, was used as a control. Treatment of JT9 cells with anti-NKTa resulted in a strong blockade of the cytolytic reaction against JM, whereas treatment of this target cell line with anti-TNKtar resulted in a partial but significant blockade, as previously reported.[14] Antibodies directed at unrelated structures, such as T8 on the effector cells or Ia on the target cells, have no blocking effect at all in this experimental system (data not shown).

DISCUSSION

In this study, we have examined in vitro alteration of undifferentiated circulating leukemic blasts obtained from a patient with acute myeloid leukemia Susceptibility to NK activity was investigated in parallel. Thymidine uptake was found to be very low in the fresh blasts. After 24 hours of culture, increasing fractions of leukemic cells began to enter S phase A week later, >80% of the cells had undergone differentiation, as evidenced by morphological criteria. Indeed, on day 8, <10% of the cells appeared as undifferentiated blasts, whereas 50% to 60% were granular, and 30% to 40% had a monocytoid aspect. During the first day of culture, leukemic cells which were resistant immediately after thawing became susceptible to lysis by IL2-activated NK cells. Simultaneously, membrane modifications took place since the TNKtar antigen, not present on day 0, was strongly expressed on the surface of the blasts by day 1

Table 5. Effects of Monoclonal Antibodies on Cytotoxicity Mediated by JT9 Cells Against Leukemia Blasts and JM Cells

		Leukemia Cells				
Treatment	JM	D0*	D1	D2	D4	D6
Medium	70	0	18	22	21	14
Anti-NKTa	29	0	6	6	4	4
Anti-TNK$_{TAR}$	46	0	8	11	11	6

*E/T ratio 10.1, percent specific cytotoxicity mean of triplicate cultures, SD ≤5%.

Moreover using the JT9 cloned cell line, we demonstrated that anti-TNKtar was able to block significantly the cytotoxic reaction against the cultured leukemic cells.

NK activity is operationally defined as the capacity displayed by a population of lymphocytes to destroy without apparent preimmunization a series of activated cells such as myeloid progenitors,[15] viral-transformed cells and, of course, tumor cells.[16] This spontaneous lytic activity mediated by NK cells is enhanced following short-term incubation with IL2.[17] Using a given target cell, biological mechanisms responsible for transformation from resistant to susceptible phenotype are presently unknown Therefore, the preferential susceptiblity of activated cells to natural killing is not well understood. In this respect, experiments designed to investigate the role of target cell activation in NK susceptibility cannot be performed with permanently growing hematopoietic cell lines (ie, K562).

We have had the opportunity to study fresh leukemic blasts which, in culture, undergo a rapid activation leading to subsequent proliferation and/or differentiation. The triggering stimulus responsible for these in vitro alterations may have been DMSO, since blasts were cryopreserved prior to being studied. It is also conceivable that circulating blasts were undifferentiated because of in vivo suppression mechanisms. Fresh leukemic cells were found to be resistant to both resting and IL2-enhanced natural killing. In contrast, the blasts became clearly susceptible to this latter type of cytotoxicity during in vitro culture. However, susceptibility to IL2-activated NK cells was acquired prior to an increase in DNA synthesis and subsequent proliferation, and before further steps of morphological differentation were reached. Consequently it is evident that, in the present experimental system, acquisition of susceptibility did not correlate with a stage of further differentiation but with an early step of cell activation that would eventually lead to it. Furthermore, there was a positive correlation between susceptibility and early surface expression on target blasts of TNKtar antigen.[14]

TNKtar is a 40 to 80-kd heterodimeric structure previously shown to be identical to 4F2.[18,19] It is found at various densities on all human cultured cell lines of hematopoietic and extra hematopoietic origin that have been tested thus far. This molecule is present with a very low density on normal lymphoid cells but is strongly expressed within 24 hours after cell activation triggered, for example, by mitogens. We have shown that TNKtar also represents an activation-dependent surface antigen on myeloid cells that had not been previously established. TNKtar was identified in earlier studies[14] as one target structure "seen" on cultured hematopoietic cell line membranes by a series of NK clones including JT9, which has been described in detail.[6,7,14] JT9 cells have a mature T cell phenotype coexpressing T1, T3, T8, T11, and T12 antigens. Moreover, they possess a clonotypic receptor termed NKTa[7] which is functionally and biochemically analogous to Ti molecules.[20,21] However, as opposed to conventional CTL, JT9 cells express NKH1A, like all NK cells,[12] and kill genetically unrelated hematopoietic tumor cell lines, including K562, in an MHC-independent fashion. Data presented here further substantiate the hypothesis that TNKtar functions as a receptor recognized on target cells by JT9 cloned lymphocytes, since anti-TNKtar blocked their cytotoxic activity against the present leukemic blasts which have not been cultured long-term. It is perhaps more important, that an unique positive correlation could be established, using a population of fresh tumor cells, between acquisition of susceptibility to JT9 and concomitant surface expression of TNKtar. It is therefore suggested that, during the process of target cell activation, TNKtar expression may represent a necessary signal for recognition and subsequent lysis.

As shown in these studies, LGL fractions and all NK clones tested capable of killing the leukemic blasts reached a maximal level of cytotoxicity within 24 hours of culture. This observation is of interest in light of the heterogeneity of human NK cells. Indeed, it is now well established that natural killing is mediated by distinct and probably multiple populations of cytotoxic lymphocytes.[22-27] The development of human NK clones has helped[4-7] to demonstrate this heterogeneity and, more important, has helped to dissect it because monoclonal populations of cells can be analyzed by this approach. As mentioned, it was established by studying NK clones that certain $[T3/Ti]^+$ cells express NK-associated antigens such as NKH1A and behave functionally as NK cells. However, it is clear that they represent a very minor population within NK active lymphocytes which, in the great majority are T3− NKH1A+.[12] Except for JT9, all NK clones used in these studies are T3− NKH1A+ and partially reflect the heterogeneity of circulating NK cells. Indeed, they display four distinct NK-associated phenotypes characterized by a limited series of Mabs. Moreover, they may express major differences in lytic repertoire[6] although they cannot be distinguished phenotypically. For example, JT3 and TC59 cells are both T3−, T4−, T8−, T11+, NKH1A+, and NKH2+ (Table 1A), and they kill K562, and JM cell lines (Table 1B). However, JT3 has very little cytotoxicity against leukemic blasts, whereas TC59 is strongly active against these latter tumor cells (Table 4).

The structure(s) recognized on target cell membranes by individual populations of T3−, NKH1A+, NK active lymphocytes is unknown. It is certainly not TNKtar antigen (or a close epitope) because the corresponding antibody does not block cytotoxic reactions mediated by a number of representative clones. Yet it remains to be determined whether target structure(s) (eg, TNKtar) of $[T3/Ti]^+$, $NKH1A^+$ cells such as JT9, and target structure(s) of $T3^-$ $NKH1A^+$ cells are either distant regions of the same molecule or are distinct antigens linked to cell activation or, alternatively, totally unrelated structures. The present data suggest that the latter hypothesis is unlikely. We found that in spite of the phenotypic and functional heterogeneity of NK cells, LGL fractions and all active clones became lytic at the same time during in vitro alteration of the leukemic cells. Meanwhile, cold target inhibition experiments indicated that lack of initial cytotoxicity by individual populations of T3−, NKH1A+ cells was probably due to a binding defect. These results strongly suggest that target structure(s) for at least a fraction of T3−, NKH1A+ NK active lymphocytes are expressed very early and with kinetics similar to those of

TNKtar in the process of target cell activation. Consequently, analysis of the present experimental system supports the view that the search for currently undefined NK target structure(s) should further focus on the identification and characterization of early activation antigens.

Multiple studies have pointed out that NK cells may play an important role in host defense against tumors.[16] The data presented here suggest that a better understanding of NK target cell interaction mechanisms should help to delineate strategies of immunomanipulation. For example, in this patient, both activation of circulating blasts by an appropriate drug and enhancement of NK cell cytotoxicity by IL2 would have been required for the design of a potentially beneficial adjuvant therapy.

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Diversity of Human γ-Globin Gene Loci Including a Quadruplicated Arrangement

By Shunichi Shimasaki and Iwao Iuchi

A quadruplicated γ-globin gene as a (5′-$^G\gamma$-γ-γ-$^A\gamma$-3′) was detected in an adult Chinese during a survey designed to detect $^G\gamma$- and $^A\gamma$-globin genes in Japanese. Five triplicated (-$^G\gamma$-γ-$^A\gamma$-) and two single (-γ-) haplotypes were also detected in 103 healthy adult Japanese. All of the unusual chromosomes appeared to reflect an unequal but homologous crossover between $^G\gamma$- and $^A\gamma$-globin genes. A new *Bgl* II polymorphic site located around the 3′ terminal region of the $^G\gamma$-globin gene was also discovered.

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THE DUPLICATED human fetal globin genes encode different forms of γ-chains designated as $^G\gamma$ and $^A\gamma$. The two genes originate by duplication of five kilobases (kb) of DNA, either through a simple unequal recombination of two small preexisting repetitive elements or through a more complex series of events not requiring any preexisting repeats.[1]

Based on a comparison of the DNA sequences of the two γ-globin genes, the occurrence of interchromosomally unequal but homologous crossovers between $^G\gamma$- and $^A\gamma$-globin genes (gene conversion) has been strongly suggested.[1,2] An unusual arrangement of the γ-globin gene locus in a chromosome has also been reported in some racial groups,[3,4] as expressed in a haploidy formula of -γ-γ-γ-/ or -γ-/.

We examined the arrangement of γ-globin genes to learn the frequency of intergenic crossover in Japanese. The results of that survey are reported, with special emphasis on the discovery of a quadruplicated γ-globin gene haplotype (four γ-globin gene loci on one chromosome)

MATERIALS AND METHODS

Routine hematologic studies were carried out by standard methods [5] Conventional methods were used for hemolysate preparation and fetal hemoglobin (Hb F) determination.[6] Blot hybridization studies were performed with DNAs of 104 healthy unrelated adults (103 Japanese and one Chinese)

A DNA sample of high mol wt was isolated from leukocytes collected from 10 mL of venous blood by standard methods [7] Four micrograms of each sample were digested with 20 U of *Bgl* II for five hours under conditions recommended by the manufacturer (Takara Shuzo Co, Ltd, Kyoto, Japan). Cleaved DNA fragments were precipitated with ethanol and redissolved in 30 μL of TE (10 mmol/L of Tris-HCl, 1 mmol/L of EDTA, pH 8 0) buffer. The DNA digest was electrophoresed on a 0 6% agarose gel (pH 8.0) and transferred onto a nylon membrane (Zeta-Probe, Bio-Rad, Richmond, Calif) by Southern blotting method.[8]

The DNA fragments on the membrane were hybridized to a mixture of three radioactive probes: (a) a 3.4-kb *Hind* III fragment containing the $^A\gamma$-globin gene; (b) a 2 3-kb *Pst* I fragment containing the δ-globin gene; and (c) a 3.8-kb *Pst* I-*Bgl* II fragment containing the β-globin gene. Each of the DNA probes was derived separately from recombinant plasmids pγ 3.4,[9] *Pst* δ (pHδ_1)[9] or *Pst* β[10] isolated individually from transformed *E coli* HB 101 under P2-EK1 containment conditions in accordance with the guidelines of the Ministry of Education, Science and Culture of Japan.

The probe fragments were nick-translated[11] in the presence of α-^{32}P dCTP to convert them into a radioactive form. Their specific activity was in a range of 1 to 3×10^8 dpm/μg After hybridization, the nylon membranes were washed stringently, dried, and exposed to x-ray film at −70 °C for 15 hours according to the protocols described by Goossens and Kan.[12]

DNA samples that showed an abnormal pattern on autoradiograms were further digested by other restriction endonucleases including *Bcl* I, *Xmn* I, *Pst* I, *Xba* I, *Bam* HI, and *Eco* RI, followed by the same analyses except for hybridization to an individual probe.

RESULTS

Bgl II cleaves human DNA to produce the linked γ-globin gene ($^G\gamma$ and $^A\gamma$) loci and each of the δ-globin and β-globin gene loci. Autoradiograms obtained by a mixture probe demonstrated clear bands certifying complete DNA digestion with *Bgl* II and providing an accurate estimation of the DNA fragment sizes (Fig 1).

The autoradiograms of 104 individuals obtained by *Bgl* II digestion and use of the mixture of probes revealed 95 instances carrying the DNA bands of 13, 8.2, and 5.0 kb with approximately equal intensity on scanning. They could be accordingly classified into the most common genotype (group N in Fig 1). The other nine DNA samples, however, showed a clear but fainter 13-kb band than did those of 8.2 and 5.0 kb (groups A, B, C, and D in Fig 1). In addition, seven of these nine samples showed an extra restriction fragment. DNA samples from these nine individuals were accordingly classified into four groups: A (5 cases), B (one case), C (2 cases), and D (one case) based on the band pattern

In order to identify these extra bands, hybridization to each probe was carried out individually; only the γ-globin specific probe hybridized to each of the extra bands (Fig 2). Moreover, additional extra bands (8.1 kb) were observed in the samples of group C which had not been recognized as containing an extra band when hybridized to the mixture of probe.

The sizes of the extra bands were estimated to be 18, 23, and 8.1 kb in groups A, B, and C, respectively, and 10 and 3.2 kb in group D. It was evident that the 8.1-kb band in group C was superimposed on the 8.2-Kb *Bgl* II band derived from the δ-globin gene by hybridization to the mixture of probes.

The nine DNA samples were digested with *Bcl* I. Autoradiograms detected the 20-kb fragment commonly seen due to digestion outside the linked γ-globin gene loci in each and an extra fragment of varied size in all samples except for the

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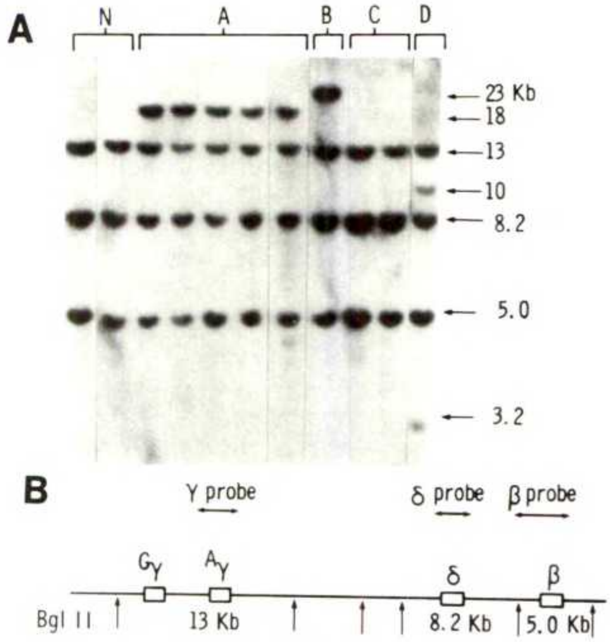

Fig 1. (A) Autoradiogram of *Bgl* II digestion patterns. Probe: $\gamma + \delta + \beta$; n, usual 95 individuals. A, B, C, D are groups with an unusual band pattern. (B) *Bgl* II restriction sites in a usual non-α-globin gene cluster.

individual of group D (Fig 2). The difference in the size of these extra fragments from the normal 20-kb fragment was the same as the difference between the extra bands observed with *Bgl* II digestion and the usual 13-kb *Bgl* II fragment. It was consequently considered that the 13-kb *Bgl* II fragment contained: (a) an additional 5-kb DNA in group A; (b) an additional 10-kb DNA in group B; (c) a deletion of 5-kb DNA in group C; and (d) an additional *Bgl* II restriction site dividing the usual 13-kb fragment into 10-kb and 3.2-kb fragments in group D. This phenomenon was further confirmed by digestion with *Xmn* I, which cleaves human DNA to produce the linked γ-globin gene as shown in Fig 3. When the genomic DNA of group N was digested with *Xmn* I and hybridized to a γ probe, three types of fragment patterns were observed (Table 1) in accordance with a polymorphic *Xmn* I site at 157 to 166 base pairs (bp) 5′ to the Cap site of the ${}^{G}\gamma$-globin gene (Fig 3).[13] In groups A, B, and C, extra bands with an additive or a reductive size of 5 kb or 10 kb as compared to the common bands were observed as seen in *Bgl* II digestion (Table 1).

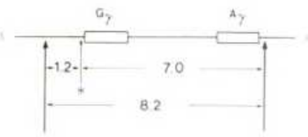

Fig 3. *Xmn* I restriction sites around ${}^{G}\gamma$- and ${}^{A}\gamma$-globin genes. Asterisk denotes the *Xmn* I polymorphic site. Sizes in kilobases.

The DNA samples were then digested with *Pst* I, which cleaves at the site of the codon corresponding to the 136th amino acid of the ${}^{A}\gamma$-globin chain (third exon) but not at the same position in the ${}^{G}\gamma$-globin gene. The autoradiograms obtained with a γ probe revealed the three normal bands (Fig 4). The relative intensities of the intergenic 4.9-kb fragment between ${}^{G}\gamma$- and ${}^{A}\gamma$-globin gene was compared to the two bands of 4.0 kb and 2.8 kb derived from the upstream region of the ${}^{G}\gamma$-gene and the downstream region of ${}^{A}\gamma$-gene, respectively. The ratios were higher in groups A and B and lower in group C than were those of the common group. Namely, the relative intensities of the 4.9-kb band to 4.0-kb band were 1.4, 1.8, 2.5, 0.8, and 1.4 for groups N, A, B, C, and D, respectively.

Xba I, *Bam* HI, and *Eco* RI were also used to characterize these unusual DNAs. All of the intergenic DNA fragments shown with arrows in Table 2 demonstrated the same tendency as did *Pst* I digestion. For example, the *Xba* I digestion showed the relative intensities of the intergenic 4.9–7.5-Kb fragment as 1.9, 2.5, 3.3, 1.4, and 1.9 for groups N, A, B, C and D, respectively. Restriction endonuclease maps around the fetal globin genes of these unusual chromosomes were established by these findings, as in Fig 5.

Two different-sized fragments of group B observed by the *Bgl* II, *Bcl* I, and *Xmn* I digestion were thought to come from the heterozygosity of the γ-globin gene cluster. The individual in group B was therefore considered to be heterozygous for the quadruplicated γ-globin gene rather than homozygous for a chromosome with three γ-globin genes. The individuals of groups A and C were heterozygous for triplicated and single γ-globin genes, respectively. The frequen-

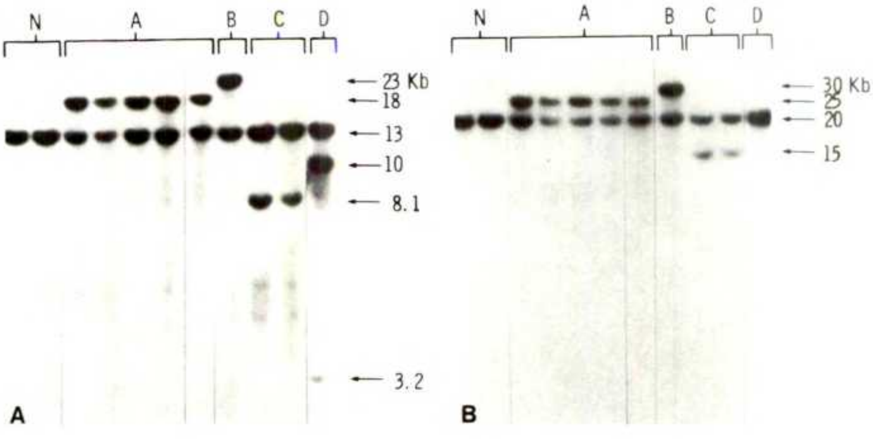

Fig 2. Autoradiograms of *Bgl* II (A) and *Bcl* I (B) digestion patterns. Probe, γ; n: usual individuals; A, B, C and D are groups with an unusual γ-globin gene.

Table 1. Size (in Kilobases) of the Fragments Digested with *Xmn* I

	Group				
No. of Case	N	A	B	C	D
1	8.2	13, 8.2	18, 8.2	8.2, 3.3	8.2
2	8.2, 7.0, 1.2	13, 8.2	—	7.0, 2.1, 1.2	—
3	7.0, 1.2	13, 8.2	—	—	—
4	—	13, 8.2	—	—	—
5	—	13, 8.2	—	—	—

Table 2. Size (in Kilobases) of Restriction Fragments in the Region of a Fetal Globin Gene

Group	*Xba* I	*Bam* HI	*Eco* RI
N	7.5, 4.9, 3.7	15.5, 4.9, 2.6	7.2, 2.6, 1.7
A	7.5, 4.9↑, 3.7	15.5, 4.9↑, 2.6	7.2, 2.6↑, 1.7↑
B	7.5, 4.9↑, 3.7	15.5, 4.9↑, 2.6	7.2, 2.6↑, 1.7↑
C	7.5, 4.9↓, 3.7	15.5, 4.9↓, 2.6	7.2, 2.6↓, 1.7↓
D	7.5, 4.9, 3.7	15.5, 4.9, 2.6	7.2, 2.6, 1.7

↑: Increased intensity; ↓: decreased intensity.

cies of the triplicated γ-globin haplotype and the single one in this survey were 0.024 and 0.010, respectively.

The nine individuals of groups A, B, C, and D showed no hematologic abnormalities or evidence of hemolysis (RBC, PCV, Hb, MCV, MCHC, MCH, reticulocyte count, and serum bilirubin), and their Hb F of 0.2% to 0.4% were within the normal range.

DISCUSSION

A high degree of homology between the $^{G}\gamma$- and $^{A}\gamma$-globin genes extends from about 500 bp 5′ to the Cap site to a region of simple base sequence within the large intervening sequence (IVS-2).[1] This simple base sequence region seems the most likely site for an interchromosomally unequal but homologous crossover. The crossover mechanism between the $^{G}\gamma$- and the $^{A}\gamma$-globin gene may be shown as in Fig 6, based on a general model for genetic recombination proposed by Meselson and Radding.[13,14] When a branch migration from a cross-bridge site is short, and the strand cleavage occurs within the gene, a new single hybrid gene represented as -$^{G}\gamma^{A}\gamma$- is generated. When the branch migration extends upstream beyond the gene, the single γ-globin gene is an intact $^{A}\gamma$-gene. In the case of the triplicated γ-globin gene chromosome, on the other hand, the central gene generated by the crossover may be -$^{G}\gamma$- or -$^{A}\gamma^{G}\gamma$- depending on subsequent DNA replication or mismatch repair in the heteroduplex region. This haplotype is therefore expressed as 5′-$^{G}\gamma$-γ-$^{A}\gamma$-3′ without defining the central γ-globin gene.

The region of the crossover on the eight unusual chromosomes we studied was inferred from the *Pst* I and *Xmn* I analysis. The *Pst* I sites, specific for $^{A}\gamma$-genes, were not present in all of the inner genes of the triplicated and the quadruplicated γ-globin gene chromosomes, but analyses of the single γ-globin gene chromosomes clearly showed this cleavage site. These results suggest that all the crossover events occurred upstream from the *Pst* I site in the 3rd exon, of the $^{A}\gamma$ gene.

In the *Xmn* I analysis, the appearance of the extra fragments of 13 and 18 kb in groups A and B, respectively, suggests that the crossover occcurred within the normal 8.2-kb *Xmn* I fragment. The left *Xmn* I site defining the 8.2-kb fragment in Fig 3 is at 1.3 Kb upstream from the Cap site of the $^{G}\gamma$-globin gene. The crossover region of groups A and B, therefore, occurred between the *Xmn* I site 1.3 kb 5′ to

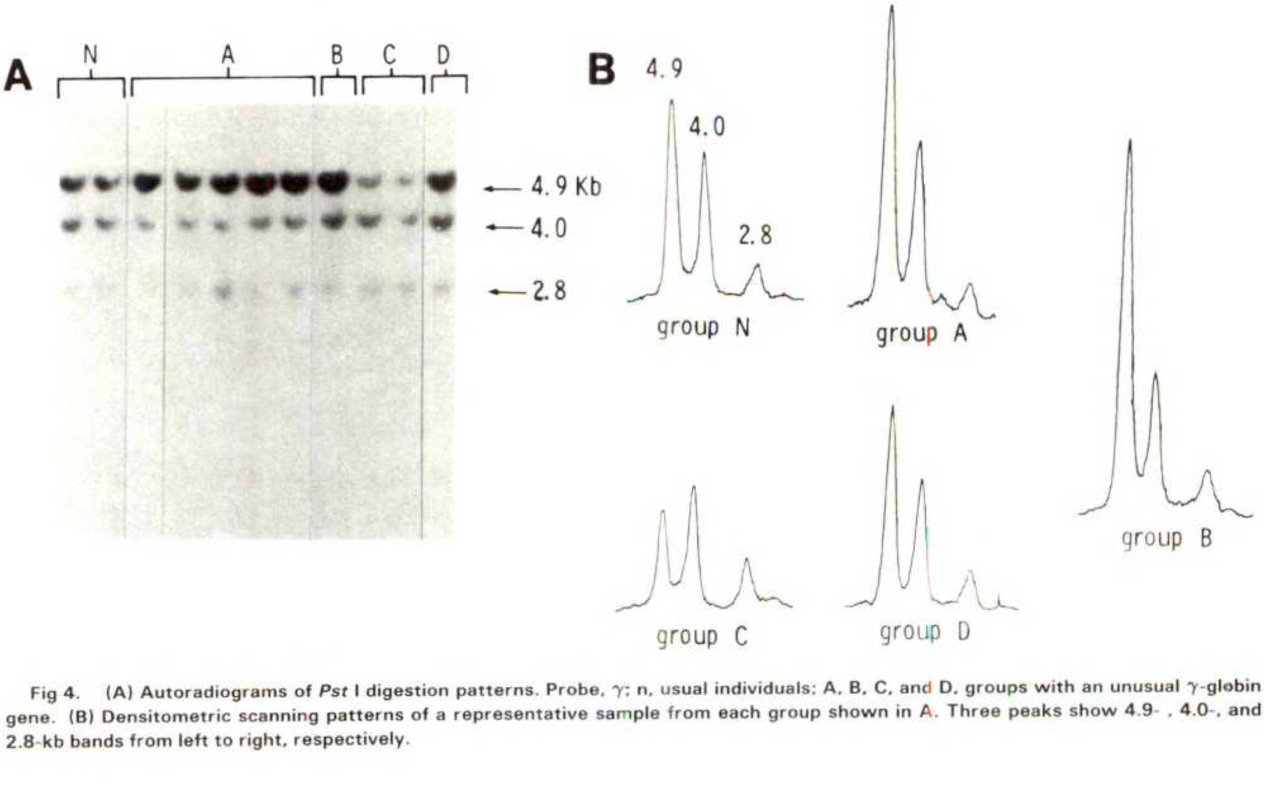

Fig 4. (A) Autoradiograms of *Pst* I digestion patterns. Probe, γ; n, usual individuals; A, B, C, and D, groups with an unusual γ-globin gene. (B) Densitometric scanning patterns of a representative sample from each group shown in A. Three peaks show 4.9- , 4.0-, and 2.8-kb bands from left to right, respectively.

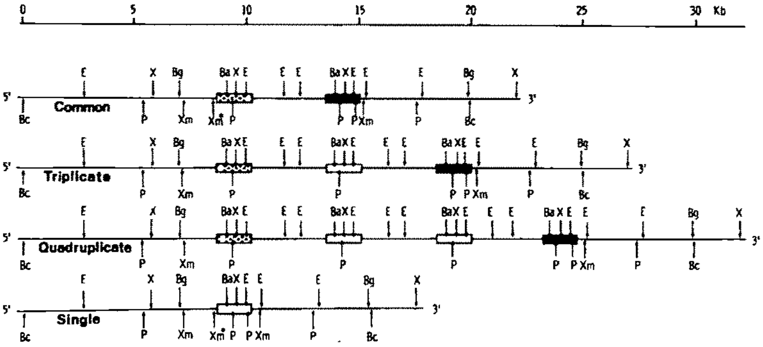

Fig 5. Gene arrangement and restriction endonuclease maps of usual and unusual γ-globin gene clusters. Asterisk represents *Xmn* I polymorphic restriction site. Stippled and hatched rectangular boxes are $^G\gamma$- and $^A\gamma$-globin gene, respectively. Open rectangular boxes are γ-globin genes generated by cross-over. Ba, *Bam* HI; Bc, *Bcl* I; Bg, *Bgl* II; E, *Eco* RI; P, *Pst I*; X, *Xba* I; Xm, *Xmn* I.

the Cap site of the $^G\gamma$ gene and the *Pst* I site in the 3rd exon of the $^A\gamma$ gene.

Formation of these hybrid chromosomes by interchromosomal crossovers occurs within the region proposed by Shen et al.[1] The quadruplicated γ-globin gene haplotype may have arisen through an unequal but homologous crossover between the triplicated and the usual duplicated haplotypes or between two triplicated haplotypes. It is accordingly represented as 5′-$^G\gamma$-γ-γ-$^A\gamma$-3′.

The process of unequal but homologous crossover produces an equal number of chromosomes with a triplicated γ-globin gene locus and a single one. However, the frequency of the triplicated γ-globin gene chromosome was twice as great as that of the single γ-globin gene chromosome in this survey. It is unlikely to suppose that a single γ-globin gene haplotype confers a serious selective disadvantage; two newborns who were homozygous for a single γ-globin gene have been reported without severe γ-thalassemia.[15] The number of γ-globin gene loci does not affect Hb F production in adults since the Hb F levels were within normal limits.

This article provides a description of a *Bgl* II polymorphic restriction site near the globin genes. One individual in group D demonstrated a site that split the usual 13-kb fragment containing the $^G\gamma$- and $^A\gamma$-globin genes into 10-kb and 3.2-kb fragments. The probe used in this hybridization was an $^A\gamma$ *Hind* III 3.4-kb fragment which could also hybridize to the homologous $^G\gamma$-gene sequence. If the new polymorphic *Bgl* II site is located 3.2 kb 3′ to the leftward *Bgl* II site of the 13-kb fragment shown in Fig 1, it would be observed on the autoradiogram. On the other hand, if the 3.2-kb fragment derived from the opposite end of the 13-kb fragment, it would not be expected to hybridize to the γ probe. The observed 3.2-kb fragment was, therefore, formed by a new *Bgl* II site near the 3′ terminal region of the $^G\gamma$-globin gene.

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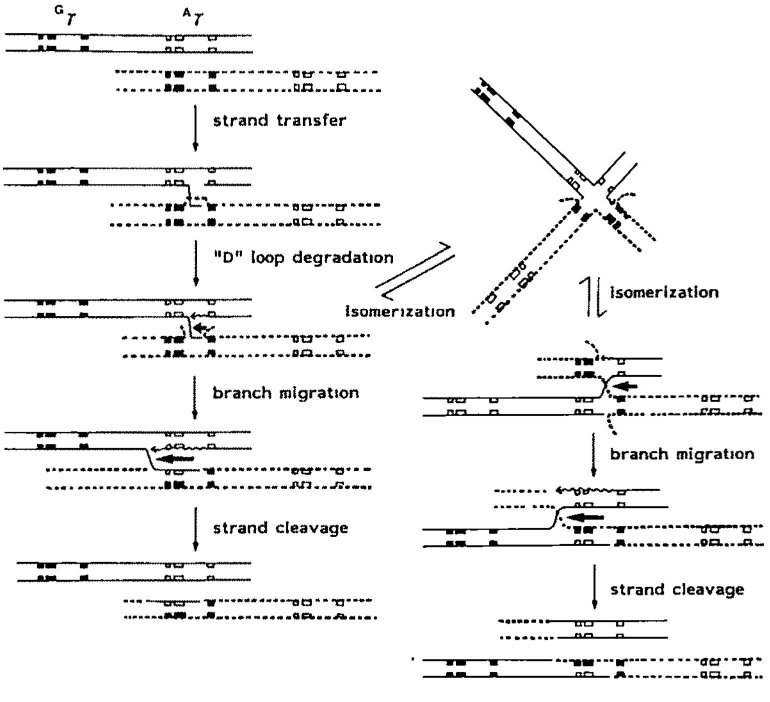

Fig 6. Possible recombination model between $^G\gamma$-globin and $^A\gamma$-globin genes. The black and white boxes represent the globin coding region. The wave lines with an arrow denote a replicating DNA strand.

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Effect of Acidic and Basic Isoferritins on In Vitro Growth of Human Granulocyte-Monocyte Progenitors

By Laura Dezza, Mario Cazzola, Wanda Piacibello, Paolo Arosio, Sonia Levi, and Massimo Aglietta

Acidic isoferritins have been previously found to be highly potent inhibitors of hematopoietic progenitors at concentrations of 10^{-16} to 10^{-18} mol/L, and it has been suggested that acidic isoferritin inhibitory activity plays a role in the regulation of normal hematopoiesis and also in the pathogenesis of leukemia. To characterize the ferritin species that affect the in vitro growth of human colony-forming unit–granulocyte-macrophage (CFU-GM), we tested different preparations of basic (L-subunit–rich) and acidic (H-subunit-rich) isoferritins. Three preparations of human liver (basic) ferritin did not show any effects on CFU-GM growth at concentrations up to 10^{-9} mol/L, irrespective of the degree of glycosylation. Acidic isoferritins were purified both from HeLa cells and human heart. HeLa cell ferritin did not affect in vitro colony formation. One of two preparations of human heart ferritin, containing 5% glycosylated ferritin, showed a mean inhibition of 26% ± 8% of the control at 10^{-9} mol/L ($P < .02$), whereas the other preparation, which contained no glycosylated ferritin, did not show any effect of CFU-GM growth. A preparation enriched for glycosylated acidic isoferritins from human heart was found to produce a mean inhibition of 32% ± 11% of the control at 10^{-9} mol/L ($P < .01$), whereas another one was ineffective. A significant part of the inhibitory activity was removed by preincubation with the monoclonal antibody 2A4 directed against human heart ferritin. The present findings indicate that basic isoferritins, ie, the predominant ferritin type in human blood, have no effect on the growth of human CFU-GM, and this is in keeping with indirect clinical evidence. Inhibition of colony formation may be obtained by some preparations of acidic isoferritins that are rich in H subunits and bind to concanavalin A. The mechanism(s) responsible for this are not clear, but the effective concentrations are higher than those found in human blood both under normal conditions and in leukemia. At present, the physiologic significance of the observed inhibitory activity is uncertain.

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FERRITIN is ubiquitous in all cells, and its major functions are iron storage and detoxification.[1,2] The structural and immunologic properties of this molecule vary from one tissue to another and are related not only to the iron status but also to other factors including cell differentiation and malignant transformation.[3,4] Multiple forms of ferritin occur in most cells, with all molecules consisting of 24 subunits arranged as hollow protein shells having an approximate molecular weight of 500,000.[3] These isoferritins are families of heteropolymers fashioned from different proportions of two distinct subunit classes, heavy (H) and light (L), with molecular weights of approximately 21,000 and 19,000, respectively.[3] The L-subunit–rich isoferritins are basic (high pI) and predominate in iron storage organs (liver and spleen) and plasma, whereas the H-subunit–rich isoferritins are acidic (low pI) and occur in tissues of low nonheme iron content (heart, erythroid cells, monocytes, lymphocytes) and in fetal and some malignant tissues. This model for tissue ferritin phenotypes based on two distinct subunit species has been validated by the demonstration that the two subunits are encoded by distinct messenger RNAs (mRNAs)[5] and recent evidence for the existence of at least two ferritin genes.[6–8] However, the matter could be more complex because a glycosylated G subunit (molecular weight about 23,000) has been isolated from human serum.[9] It is possible to evaluate basic and acidic isoferritins separately using immunoassays based on monospecific antibodies raised against L-subunit–rich and H-subunit–rich molecules.[10]

Although ferritin function is inextricably linked to iron metabolism,[11] there has been evidence of a role for ferritin in lymphocyte migration[12] and T cell function[13] as well as in the control of myelopoiesis. In fact, a leukemia-associated inhibitory activity (LIA) directed against human granulocyte-monocyte progenitors[14] was identified by Broxmeyer and his associates as acidic isoferritins.[15] Acidic isoferritin inhibitory activity (AIFIA) was later found in bone marrow and blood cells of normal donors,[16] and a specific role for monocyte-macrophage–derived acidic isoferritins as feedback regulators of hematopoietic progenitor cells was suggested.[16] However, this has been disputed because of certain inconsistencies with the present picture of human isoferritins.[17]

In this work, we have studied the effects of different isoferritins on the growth of cultured granulocyte-macrophage precursor cells and correlated these effects with biochemical and immunologic characteristics of the ferritin tested.

MATERIALS AND METHODS

Purification of human liver, heart, and HeLa cell ferritin. Eight ferritin preparations were made and coded as A, B, C, D, E, F, G, and H. The main characteristics of these isoferritins are reported in Table 1.

The procedure for purification of human liver ferritin (preparations A and B) was essentially the same as previously described in detail by Arosio et al.[18] It consisted of heat extraction at 75 °C, ammonium sulfate precipitation (60% saturation), precipitation at

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Table 1. Biochemical and Immunologic Characteristics of the Isoferritin Preparations

Name	Origin	Relevant Purification Steps	Purity (%)	Immunologic Reactivity* Liver Type (%)	Immunologic Reactivity* Heart Type (%)	Binding to Con A (%)
A	liver	crystallization	>97	100	0	2
B	liver	crystallization	>97	100	0	2
C	heart	passage through Con A–Sepharose	95	15	85	0
D	heart	preparative electrophoresis; no passage through Con A–Sepharose	>97	14	86	5
E	HeLa cells	as in D	>97	9	91	0
F	heart	no heat extraction; elution from Con A–Sepharose with 0 1 mol/L α-methyl mannoside	<5	46	54	100
G	heart	tissue heated at 75 °C and then as in F, ultracentrifuged	<5	49	51	100
H	liver	as in G	~10	98	2	100

Abbreviation: Con A, concanavalin A.

*Ferritin concentration was evaluated with immunoradiometric assays based on polyclonal antibodies against human liver ferritin and the monoclonal antibody 2A4 against human heart ferritin; each value was then expressed as a percentage of the total ferritin concentration

300,000 g for 90 minutes, gel filtration on a Sepharose 4B column, and crystallization with 5% cadmium sulfate.

Human heart (preparation D) and HeLa (preparation E) ferritins were purified by similar procedures except that the crystallization step was omitted and replaced with diethyl aminoethyl chromatography and preparative gel electrophoresis on a polyacrylamide gradient (10% to 30%) gel slab

Human heart preparation C was purified with a similar procedure except that the heated homogenate was passed through a column of Affi gel Blue (Bio-Rad Laboratories, Richmond, Calif); equilibrated in 20 mmol/L Tris-HCl, pH 7.4, and on a column of Con A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden); and the break-through peaks collected. For this preparation, the preparative gel electrophoresis step was omitted.

Isoferritin preparations were analyzed by means of isoelectrofocusing, sodium dodecyl sulfate (SDS) electrophoresis, and immunoblotting as previously described.[10,18,19] Preparations A, B, D, and E appeared pure by native and SDS gel electrophoretic analyses, whereas preparation C contained 5% contaminants. The ferritin protein concentration was determined by the method of Lowry et al [20]

Glycosylated ferritins were obtained by loading nonheated tissue homogenates (preparations F and H) or heated homogenates (preparation G) on a Con A–Sepharose column. After extensive washing, glycosylated proteins were eluted with 0.1 mol/L α-methyl glucoside. These preparations were concentrated either on an Amicon M 300 membrane (preparation F and H) (Amicon Corp, Danvers, Mass) or by precipitation at 300,000 g for 90 minutes (preparation G). The ferritin concentration was detected by immunoassays In ferritin preparations F and G, ferritin did not account for more than 5% of the total protein, whereas preparation H was somewhat more than 10% pure.

Monoclonal antibody to heart ferritin. The monoclonal antibody 2A4 against human heart ferritin was developed by Cavanna et al,[21] and its characteristics were reported by Arosio et al.[10]

Immunoassays for liver and heart ferritin. The two-site immunoradiometric assay for human liver ferritin was prepared as previously described.[22] The two-site immunoradiometric assay for heart ferritin was prepared as described by Cavanna et al[21] using the monoclonal antibody 2A4

Binding to Con A. This was determined as previously detailed.[10]

CFU-GM assay procedures Normal bone marrow was obtained from normal volunteers or from patients undergoing chest surgery for nonmalignant diseases. Bone marrow cells were collected in preservative-free heparin (50 to 100 U/mL) A quantity of 1×10^5 low-density bone marrow cells, separated as previously described,[23] were plated in a 1-mL mixture containing Iscove's modification of Dulbecco's medium (IMDM), 15% fetal bovine serum, 0.3% agar (Difco Laboratories, Detroit, Mich), and 10% conditioned medium from the 5637 cell line as a source of colony-stimulating activity (CSA) in the presence or absence of the various isoferritins. Sodium azide was removed from the isoferritin preparations by dialysis prior to the cultures. Preliminary studies showed that ferritin concentrations in the 5637-conditioned medium were below the lower detectability limit of both the liver ferritin and 2A4 assays Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Plates were scored for colonies after seven days of incubation, a time that has been shown previously to be optimal for the demonstration of inhibition of CFU-GM from normal donors by AIFIA.[14,15] All aggregates containing more than 40 cells were counted as colonies.

To study the neutralization of a possible isoferritin-associated inhibitory activity by a specific antibody, active isoferritin preparations were preincubated at 10^{-8} mol/L with the monoclonal antibody 2A4 prior to the culture. The final dilution of the ammonium sulfate precipitate of the 2A4 ascitic fluid was calculated so as to have an excess of antibody as compared to ferritin molecules

The bone marrow culture studies were carried out in a blind fashion. The various isoferritin preparations were made by two of us (S.L. and P.A.) and marked with different letters (A to H). People working with the CFU-GM assays did not know the content of such preparations Two plates were scored for each sample by two independent investigators, and the mean value of the four scores was considered the result of that single experiment. Colony growth in the presence of different preparations was expressed as a percentage of the control, and the results of different experiments were expressed

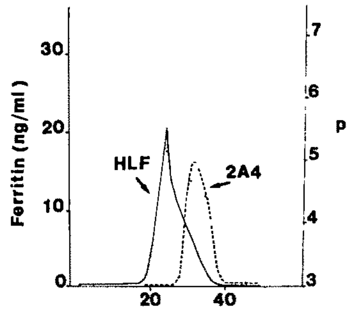

Fig 1. Isoferritin analysis of the heart ferritin preparation G containing only glycosylated isoferritins eluted from a Con A–Sepharose column with 0.1 mol/L α-methyl mannoside. In this analysis, isoferritins were separated on polyacrylamide gel isoelectrofocusing.[17] The bands containing isoferritins were cut and eluted. Then, on each sample, the pH was measured, and the ferritin concentration was evaluated by means of immunoradiometric assays based on polyclonal antibodies against human liver ferritin (HLF) and the monoclonal antibody 2A4 against human heart ferritin. Values for pH (· · · · · ·) and concentrations of liver-type (——) and heart-type (----) ferritin of different fractions are reported.

as means ±1 SD. Differences between means were determined by using the *t* test for paired observations after logarithmic transformation of the original colony scores (number per plate).

RESULTS

Isoferritin preparations. Table 1 summarizes the biochemical and immunologic characteristics of the ferritin preparations used in the following experiments. Liver ferritin preparations A and B did not show any significant differences in their isoelectrofocusing profiles or subunit composition as judged by SDS electrophoresis. Biochemical analyses of heart preparations C and D showed that these isoferritins had about 85% H subunits; their isoelectrofocusing profiles covered a large spectrum of isoferritins and were not distinguishable. The only difference between preparations C and D was that a minor portion (5%) of ferritin D bound specifically to Con A whereas ferritin C was 100% nonglycosylated. HeLa cell ferritin (preparation E) had approximately the same proportion of H subunits as heart ferritin and did not bind to Con A.

Preparations F and H could be characterized only immunologically because of the low level of ferritin purification, and the results of these studies are reported in Table 1. One major question addressed in this work was the relationship among subunit composition (immunologic reactivity), glycosylation, and pI. Figure 1 shows the results of isoelectrofocusing studies with the heart preparation G. A large number of isoferritin bands were seen, ranging in pI from 5.8 to 4.2. Immunoassays revealed two different isoferritin populations: (1) those detected by the 2A4 immunoradiometric assay and therefore containing more than 60% H subunits had a peak value of 4.6 for pI; (2) those detected by the immunoradiometric assay based on polyclonal antibodies against human liver ferritin and therefore rich in L subunits had a peak value of 5.2 for pI.

Effect of different isoferritins on CFU-GM growth. Results of these studies are reported in Table 2. Irrespective of the degree of glycosylation, the three preparations of human liver ferritin (A, B, H) did not show any effect on CFU-GM growth at concentrations equal to 10^{-9} and 10^{-12} mol/L. HeLa cell ferritin (preparation E) also did not affect colony formation. Of the two preparations of heart ferritin, preparation C, which had been passed through a Con A–Sepharose column to remove glycosylated proteins, did not show any effect on CFU-GM growth, whereas preparation D, containing 5% glycosylated ferritin, showed a significant inhibitory effect, mainly at 10^{-9} mol/L. Of the two heart preparations containing glycosylated isoferritins (F and G), G was found to produce a mean inhibition of 32% ± 11% of the control at

Table 2. Effects of Different Isoferritin Preparations on In Vitro CFU-GM Growth

Preparation (No of Experiments)	Molarity	Origin	Colony Formation as Percentage of Control Medium (Mean ± 1 SD)	Comparison With Control Medium*	
				t	*P*
A(4)	10^{-12}	liver	100 ± 8	0.01	NS
A(4)	10^{-9}	liver	98 ± 9	0.44	NS
B(4)	10^{-12}	liver	99 ± 4	0.31	NS
B(4)	10^{-9}	liver	93 ± 8	1.82	NS
C(4)	10^{-12}	heart	98 ± 10	0.43	NS
C(4)	10^{-9}	heart	87 ± 18	1.47	NS
D(4)	10^{-12}	heart	88 ± 6	3.99	<.05
D(4)	10^{-9}	heart	74 ± 8	5.52	<.02
E(4)	10^{-12}	HeLa cells	102 ± 12	0.32	NS
E(4)	10^{-9}	HeLa cells	96 ± 17	0.95	NS
F(4)	10^{-12}	heart	99 ± 8	0.27	NS
F(4)	10^{-9}	heart	95 ± 14	0.79	NS
G(4)	10^{-12}	heart	91 ± 11	1.54	NS
G(4)	10^{-9}	heart	68 ± 11	5.01	<.01
H(4)	10^{-12}	liver	101 ± 15	0.12	NS
H(4)	10^{-9}	liver	96 ± 8	1.06	NS

Abbreviation. NS, not significant.

*Comparison was carried out by applying the *t* test for paired observations on colony scores after logarithmic transformation. Colony scores of the control medium ranged from 59 to 499 per plate.

10^{-9} mol/L, whereas F was ineffective. The major difference between these two preparations was that the step of heat extraction had been omitted with preparation F.

As reported in Table 3, a significant part of the inhibitory activity of preparations D and G on in vitro colony formation was removed by preincubation with the monoclonal antibody 2A4 directed against human heart ferritin.

Overall, nine experiments were carried out with heart ferritin preparations D and G at 10^{-9} mol/L. With ferritin D, colony formation was 70% ± 10% of the control medium, the difference being significant ($t = 5.14$, $P < .001$). With preparation G, colony formation was 68% ± 10% of the control medium, and the difference was again significant ($t = 7.40$, $P < .001$). In an attempt to investigate the reproducibility of our studies, we remade two preparations of heart ferritin according to the procedures followed with preparations D and G The new preparation D, D_1, produced at 10^{-9} mol/L an average inhibition of 24% ± 9%, which was significant ($n = 4$, $t = 4.80$, $P < .02$). Only two experiments were carried out with the new preparation G, G_1, at 10^{-9} mol/L, and colony formation was 79% and 68% of the control medium, respectively.

DISCUSSION

There is controversy surrounding the role of acidic isoferritins in the control of hematopoiesis.[11,17,24-26] According to Broxmeyer and his associates, the main characteristics of the acidic isoferritins that manifest suppressive activity upon the multipotential, granulocyte-macrophage, and erythroid progenitor cells in vitro are the following[26]: (1) they have a molecular weight of about 550,000 and a pI ranging from 4.6 to 4.9; (2) they bind to Con A and are specifically eluted by α-methyl mannoside; (3) they are inactivated by monoclonal antibodies prepared against human heart ferritin and H subunits from heart ferritin, but not by antibodies against liver ferritin; and (4) they are present in normal monocytes and macrophages and, in greater amounts, in leukemic cells.

Jacobs and his associates have noted a number of inconsistencies in the data of the Broxmeyer group and have raised the following criticisms[11,17,27]: (1) tissue ferritins, including those in monocytes and leukemic cells, are nonglycosylated or at least do not bind to Con A; (2) studies on human leukemic cell lines have shown no correlation between the ability of their extracts to inhibit human CFU-GM growth and their content in acidic, H-subunit–rich isoferritins; (3) biologic fluids, including serum, do not normally contain ferritins that react with antibodies against H-subunit–rich isoferritins; (4) AIFIA is reported to be active at concentrations as low as 10^{-18} mol/L, and this would mean approximately six molecules interacting with one cell within the target system; and (5) more important, the Jacobs group has not been able to demonstrate inhibition of granulocyte-macrophage progenitors by either heart, spleen, or serum ferritin[28]

Although inconsistencies do exist in the data of Broxmeyer and his associates,[15,16] not all the aforementioned criticisms are entirely valid.

1. Experimental evidence suggests that some 20% of ferritin subunits are made in the polyribosomes attached to the membranes of the endoplasmic reticulum.[1] This is the intracellular site at which export proteins secreted from cells are made and glycosylation takes place. It has also been suggested that some ferritin made by membrane-bound polyribosomes is assembled within vesicles and that this ferritin could be more acidic than the ferritin in the surrounding cytoplasm.[1] Shinjyo et al[29] reported fucose, mannose, galactose, and hexosamine in crystalline spleen ferritin. Previous studies in our laboratories[10] and some data of the present work show that in human liver, spleen, and heart a minor component of ferritin exists that binds to Con A and is specifically eluted with α-methyl mannoside. This fraction accounts for 2% to 5% of the total tissue ferritin and is generally richer in H subunits than is the total ferritin of the tissue extract.[10]

2. A variety of products from leukemic cells can affect in vitro granulopoiesis.[30] Consequently, there can be no simple relationship between immunologic characteristics of cell ferritin and the ability of cell extracts to inhibit colony formation.

Table 3. Effects of the Monoclonal Antibody 2A4 Against Human Heart Ferritin on the Inhibitory Activity of Acidic Isoferritins Against Human Granulocyte-Macrophage Progenitor Cells

Ferritin*	Monoclonal	No of Experiments	Colony Formation as Percentage of Control Medium (Mean ± 1 SD)	Comparison (t Test for Paired Observations)
D	none	5	68 ± 11	$t = 5\ 47$, $P < .01$ when compared with control medium
D	2A4	5	93 ± 12	$t = 4.37$, $P < .02$ when compared with D alone
none	2A4	5	100 ± 5	$t = 1.70$, $P > .05$ when compared with D + 2A4
G	none	5	67 ± 11	$t = 4.96$, $P < .01$ when compared with control medium
G	2A4	5	85 ± 13	$t = 6\ 91$, $P < .01$ when compared with G alone
none	2A4	5	100 ± 5	$t = 2.35$, $P <\ 05$ when compared with G + 2A4

With both preparations, colony formation after preincubation of the ferritin with the monoclonal 2A4 did not significantly differ from the control ($t = 2\ 30$ and $t = 1.29$, respectively, $P >\ 05$ in both cases). Colony scores of the control medium ranged from 57 to 474 per plate.

*In all experiments, the ferritin concentration was 10^{-9} mol/L.

3. Normal serum ferritin reacts with antibodies to liver or spleen ferritin, has a very low iron content, and is for the most part glycosylated.[2] Its concentration reflects the amount of storage iron, and it is believed that this protein is synthesized by the rough endoplasmic reticulum of reticuloendothelial cells and hepatocytes Acidic ferritins are present in many tissues, cells, and some biologic fluids, but cannot be detected in most sera or are present only in low concentrations.[31-33] A possible explanation for this has been recently advanced by Covell and her colleagues[34,35] and us.[33] It has been shown in fact that human serum contains binding factors for acidic ferritins, probably related to complement proteins. The interaction of H-subunit–rich ferritin with these binding factors may be responsible for the rapid clearance of tissue ferritin from circulation and the low levels of acidic isoferritins in the plasma of normal subjects. However, it should be noted that this does not rule out the possibility of a role of acidic isoferritins in local cell-to-cell interactions within the bone marrow. In fact, if acidic isoferritins had a local function, there would therefore be mechanisms to make sure that they do not accumulate in plasma and other body fluids.[35] Alternatively, as will be discussed, ferritin-binding proteins may play a role in the interaction of acidic isoferritins with cells.

4. The extremely low effective concentrations of AIFIA (10^{-16} to 10^{-18} mol/L) should not be considered biologic nonsense per se. It has indeed been found that the hematopoietic cell growth factors act at very low concentrations, and in addition, the receptor numbers per cell are extremely low.[36] There is also intriguing evidence that very few receptors need to be occupied to elicit a biologic effect, for example, as few as ten molecules of erythropoietin need to be bound to erythroid progenitors.[36] Within inhibitors of granulopoiesis, lactoferrin has been found to be effective in inhibiting colony stimulatory activity at concentrations as low as 10^{-17} mol/L by two different groups.[37,38]

In this work, we have tried to clarify the aforementioned controversy. Our findings clearly show that neither the pure form nor the glycosylated fraction of liver ferritin is able to consistently affect the in vitro growth of human CFU-GM. This is at variance with the findings of Broxmeyer et al,[15] who found that normal human liver and spleen ferritins had inhibitory activity at 10^{-10} to 10^{-15} mol/L. Our data, however, are in keeping with indirect clinical evidence. Patients with thalassemia major who are not regularly treated with desferoxamine may have serum ferritin concentrations as high as 20,000 μg/L (4×10^{-8} mol/L), about 50% of the circulating protein binding to Con A (M. Cazzola, unpublished observation). Their total white cell count and differential is either normal or may be slightly elevated, and leukopenia may be observed only as a part of the picture of severe hypersplenism.[39]

The results obtained with heart and HeLa cell ferritins are more intriguing. Only one of the two purified heart ferritin preparations and one of the two glycosylated fractions had a suppressive effect, whereas HeLa cell ferritin did not have any effect. The biochemical and immunologic analyses did not show major differences between active and inactive preparations. Only the differences in purification procedures and degree of glycosylation (Table 1) may explain, at least in part, the different effects on colony formation in vitro. The active purified heart ferritin preparation contained a minor portion (5%) of glycosylated ferritin, whereas the inactive one was fully nonglycosylated, as was the HeLa ferritin preparation. As regards the two glycosylated heart ferritin preparations (F and G), the active one (G) was heated and ultracentrifuged, whereas the inactive one (F) was not. The nonheated preparation, which is known to contain active proteases,[18] could have been partially damaged upon storage, even if phenylmethylsulfonyl fluoride had been added as a protease inhibitor. Inactivation of the suppressive activity of acidic isoferritin preparations D and G by preincubation with the monoclonal antibody 2A4 (Table 3) is one of the strongest indications that the inhibitory activity was associated with the acidic isoferritin molecules.

Although a high proportion of H subunits and glycosylation appeared to be prerequisites for the observed inhibitory effect, this activity appears to be much less potent than the previously reported AIFIA,[16] and its physiologic significance is uncertain. The inhibitory activity was detected by us at concentrations of 10^{-9} to 10^{-12} mol/L, and this means a range from 0.5 to 500 μg/L, assuming a molecular weight of 500,000. We have recently found that most normal subjects have serum concentrations of acidic ferritin lower than 1.5 μg/L (lower limit of detection)[33] and values are generally below 5 μg/L in patients with leukemia.[40] As previously pointed out, however, the low serum concentrations could be in keeping with the hypothesis of a local function.

The mechanism(s) by which acidic isoferritins exert their inhibitory activity are not clear at present. The active isoferritin preparations did not block in vitro growth of CFU-GM completely, the maximum inhibition being about 50%. This could mean that acidic isoferritins act on a subpopulation of human granulocyte-monocyte progenitors having specific receptors. For example, AIFIA has been found to suppress colony formation in vitro by an Ia-like (HLA-DR) antigen–positive subpopulation of cycling human CFU-GM.[41] Alternatively, the possibility exists that ferritin does not have a direct effect on myeloid progenitors but interacts with other active molecules There has been evidence that complement proteins may be ferritin binders in serum.[33,34] More recently, we have observed an in vitro interaction between H-subunit–rich isoferritins and the third component of human complement, C3 (V. Bellotti, P. Arosio, and M. Cazzola, unpublished observations). C3 is synthesized not only by the liver but also by macrophages and monocytes, as is the C3 proactivator, factor B.[42] Whether the interaction of acidic isoferritins and C3 can result in a reduced in vitro growth of myeloid progenitors remains to be established. Furthermore, it has been recently shown that lactoferrin can bind to ferritin, the ferritin-lactoferrin complex retaining its capacity of binding to lactoferrin receptors.[43] Since lactoferrin is able to inhibit colony stimulatory activity by binding to specific receptors on monocytes and macrophages,[37,38] the inhibitory activity of acidic isoferritins in the in vitro system could be explained by their capacity of interacting with lactoferrin.

It is hoped that a deeper insight into the effects of acidic isoferritins on in vitro growth of human myeloid progenitors will be obtained by using recombinant human homopolymers[44] and investigating the aforementioned mechanisms.

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Abnormal In Vitro Differentiation of Peripheral Blood Clonogenic B Cells in Common Acute Lymphoblastic Leukemia During Complete Remission

By R. Consolini, J. Bréard, A. Bourinbaiar, A. Goutner, V. Georgoulias, C. Canon, E. Brugerie, and G. Mathé

An in vitro B cell colony assay system was used to evaluate B cell growth from peripheral blood precursors in common acute lymphoblastic leukemia (CALL) patients in remission during maintenance therapy and in normal controls. Major differences between the two groups were found in the phenotypic and morphologic features of pooled colony cells. In both cases, the cells were E−. Controls' cells were surface immunoglobulin (sIg)-positive, and some (mean, 25%) expressed Ia determinants. By Wright-Giemsa staining, they appeared as plasmacytoid cells. In contrast, patients' cells had predominantly a lymphoblastoid appearance, fewer cells had developed sIg, and a large fraction (mean, 43%) were Ia-positive. Moreover, the CALL antigen (CALLA) was expressed by a mean of 18% (range, 2% to 72%) of the patients' colony cells, whereas CALLA was never found in control colonies. Thus, cells with immature features persist in the colonies of CALL patients. Secondary colonies could be generated from the patients' cultured cells, indicating their self-renewal capacity. CALLA+ cells were also present in the secondary colonies. Finally, cytogenetic studies showed that a fraction of the patients' colony cells had karyotypic abnormalities similar to that of the original lymphoblasts. It is believed that in CALL patients this B cell assay permits the clonal expansion of residual circulating cells linked to malignant clones that are not detectable by classic hematologic and cytologic methods.

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AN ACUTE LYMPHOBLASTIC leukemia (ALL)-associated antigen has been described by Greaves et al[1] on the surface of lymphoblasts from patients with the common variant of ALL (CALL). This antigen (CALLA) is also found in approximately one third of chronic myeloid leukemias in blast crisis as well as some T cell ALL (T-ALL) and non-Hodgkin's lymphomas (NHL).[2-4] However, despite its presence on leukemic blasts, CALLA is not leukemia specific, since it has also been detected on a small proportion of normal bone marrow (BM) cells, particularly in pediatric or regenerating marrows.[5,6] Extensive studies on the distribution of this determinant within normal tissues performed with specific rabbit heteroantisera and more recently with a murine monoclonal antibody (J5)[7] have shown a relatively broad cellular expression of CALLA, including a fraction of the cells in inflamed tonsils[8] as well as renal, gut, and breast epithelia,[9] and marrow stromal cells.[10] Within the hematopoietic lineage, however, CALLA is now considered to be a normal differentiation antigen associated with relatively immature lymphoid cells of T and mainly B lineage,[11] since it has been shown that most fetal CALLA+ cells are in early stages of B cell development and have a phenotype almost identical to that of CALL lymphoblasts.[12] The preferential expression of CALLA in early B cells, both normal and malignant, correlates with a number of recent reports that indicate that most CALL blasts belong to the B lineage.[13-17] Despite its lack of leukemic specificity, CALLA has been most useful for the differential diagnosis of leukemias along with other early differentiation markers. Its presence on a variable percentage of normal marrow cells, particularly during postchemotherapy-associated lymphocytosis,[6] prevents its use in the follow-up of CALL patients in remission. Numerous studies of the cellular distribution of CALLA have, however, shown that CALLA+ cells are never detected in the peripheral blood (PB) of healthy subjects.

We found that a small percentage of circulating CALLA+ cells were present in some CALL patients in remission receiving maintenance chemotherapy. A similar finding has been reported by Greaves et al[1] in an early study on the specificity of an anti-CALLA heteroantiserum. Such a phenotype seemed compatible with either the presence of residual leukemic cells or that of normal immature cells released inappropriately in the blood.[6] The very low representation of these cells within the PB, however, makes the determination of their nature technically difficult. We have therefore induced in vitro the clonal expansion of circulating B cells in these patients. We used a recently described technique that permits the generation of B cell colonies from both normal and malignant B lymphocyte progenitors.[18,19] The specificity of the technique resides in the exhaustive depletion of T lymphocytes from the starting population and in the use of irradiated T cells feeders together with T-conditioned medium.[18] This assay allows for the phenotypic and morphologic evaluation of the colony cells at the end of the culture. We have thus compared the differentiation of clonogenic B cells in CALL patients in complete remission (CR) to that of normal controls as well as T-ALL patients subjected to a similar maintenance chemotherapy regimen.

MATERIALS AND METHODS

Patients. A total of 20 CALL, five T-ALL, and two acute myeloid leukemia (AML) patients (aged 4 to 22 years) in CR entered the study; 16 of 18 CALL patients were in first remission, and four were in second remission. CR was checked in all cases by standard clinical and hematologic (less than 5% lymphoblasts in BM aspirates of normal cellularity) criteria. Patients were also monitored for the expression of CALLA+ cells in BM and PB CALL and T-ALL patients were on the same maintenance chemotherapy protocol (6-mercaptopurine daily, methotrexate weekly, and vindesine twice a month). A total of seven normal donors (aged 28 to 40 years) from the laboratory staff were also studied.

Cell separation Peripheral blood lymphoid cells (PBL) were separated on a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) E− cells were obtained by rosette formation with two to five aminoethylisothio-uronium bromide hydrobromide

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(AET) (Sigma Chemical Co, St Louis)–treated sheep red blood cells[20] at 4 °C for two hours followed by two successive Ficoll gradients E− cells were depleted of CALLA+ cells by complement-mediated lysis with the J5 monoclonal antibody kindly provided by Dr J. Ritz. Optimal lysis was obtained after a 30-minute incubation of 150 μL of J5 per 10^5 cells (dilution, 1/800, vol/vol in minimum essential medium [MEM, GIBCO, Grand Island, NY]) followed by a 45-minute incubation with 50 μL/10^5 cells of pretested guinea pig complement (dilution, 1/5 vol/vol, in MEM) (Institut Pasteur Production, Marne la Coquette, France) Complete removal of CALLA+ cells by this procedure was checked by indirect immunofluorescence in all cases. Treated cells were incubated with J5 antibody (1/800) and, after three washes, with fluorochrome (fluorescein isothiocyanate)-labeled goat F(ab')$_2$ antimouse Ig (Cappel Laboratories, Cochranville, Pa) The cells were then examined with a Zeiss microscope equipped with epillumination

In some experiments, E-J5− cells were further depleted of Ia+ cells, 10^5 cells were incubated with 150 μL of an anti-Ia monoclonal antibody (anti-I2) (Coulter Electronics, Hialeah, Fla) for 45 minutes at 4 °C with a subsequent incubation with guinea pig complement as described. In some cases, the presence of residual Ia+ cells, as detected by indirect immunofluorescence, led to a second complement-mediated lysis.

B cell colony assay The method described by Izaguirre et al[18] has been used In brief, 2 × 10^5 cells from both cell fractions (E− and E-J5−) were seeded in 0.8% methyl cellulose in MEM supplemented with 10% fetal calf serum (FCS) (GIBCO), 20% conditioned medium, and 3 × 10^5 normal irradiated T lymphocytes obtained from two normal donors. Conditioned medium was prepared by incubating normal T cells with 1% phytohemagglutinin (PHA, HA-16) (Wellcome) in growth medium (MEM, 10% FCS) at 37 °C in a 5% CO$_2$ atmosphere for two days. Supernatants were collected, filtered, and stored at 4 °C. Such preparations are termed PHA–T cell–conditioned medium (PHA-TCM). Normal irriadiated E+ cells were prepared from normal PBL by rosette formation with AET-SRBC, and centrifugation was done over a Ficoll-Hypaque gradient E+ cells were recovered from pellet by hypotonic lysis of the SRBC, washed twice, and irradiated with 2,500 rad from a Co source. The same T lymphocyte donors and PHA-TCM batch were used in this study Measured 0.1-mL aliquots of this preparation were distributed into the wells of a microtest plate with a flat bottom (Falcon Labware, Oxnard, Calif). The plates were incubated at 37 °C for five to seven days in a moist atmosphere with hypoxemic conditions (7% O$_2$).

Colony characterization Aggregates of more than 20 cells were counted as colonies under an inverted microscope. Individual colonies were then picked with a fine-capillary pipette containing cold phosphate-buffered saline and pooled and washed for further characterization. The cells were tested for expression of the E receptor (by SRBC rosette formation) and for the presence of surface immunoglobulins (sIg) with a fluorescein-labeled goat F(ab')$_2$ anti-human F(ab')$_2$ fragment (polyvalent) (Cappel Laboratories) The cells were also phenotyped with various monoclonal antibodies for the presence of Ia (Anti-I1) (Coulter Electronics), CALLA (J5) (donated by Dr Ritz), and a B cell–specific antigen B1 (anti-B1, donated by Dr Nadler) I1, J5, and B1 were tested by indirect immunofluorescence using fluorescein-labeled goat antimouse immunoglobulin (Cappel Laboratories) Furthermore, slides were prepared by centrifugation and stained with Wright-Giemsa for morphologic study.

Self-renewal experiments Colonies were picked, pooled, and washed, and 5 × 10^4 to 10^5 cells were replated as in the primary cultures After five to seven days, colonies were counted and characterized.

Cytogenetic analysis. Chromosome analysis was performed in the colony cells of four patients To obtain metaphases, individual colonies were picked and pooled in 0.2 mL PBS. Colcemid was added at a final dilution of 0 01 μg/mL, and cells were incubated at 37 °C for 60 to 90 minutes. Cells were exposed subsequently to a hypotonic solution with 0.075 mol/L KCl and fixed in methanol acid acetic (3:1, vol/vol) Air-dried slide preparations were made, stained with Giemsa, and examined microscopically for well-spread metaphases The chromosomal structure was studied using the G-Tzypsin Giemsa (GTG)[21] banding technique. Slide preparations were submerged in a trypsin solution (0.1%) (Sigma Chemical Co) at 22 °C for 40 to 120 seconds and stained with Giemsa The international nomenclature was used [22]

RESULTS

CALLA+ cells in BM and PB. The percentages of CALLA+ cells in BM and PB of 20 CALL patients in remission are presented in Table 1. As can be seen, more than 5% J5+ cells were detected in the BM of four of these patients. BM cellularity was normal in these cases, with no evidence of lymphocytosis. Of note is patient 11 who presented with a regenerating BM with 25% lymphocytes but had no detectable J5+ cells. The proportion of CALLA+ cells in the PB of these patients was 0% to 3% and reached 15% in one case. Removal of T lymphocytes prior to testing resulted in a minor change (1% to 2% increase) in the remaining E− population; 200 cells were examined (Table 1). PBL from five T-ALL and two AML patients as well as seven healthy volunteers were negative for J5 except for one T-ALL patient who had 2% circulating CALLA+ cells. It has recently been reported that polymorphonuclear cells react with J5.[23] However, no correlation could be made

Table 1. CALLA+ Cells in BM and PB of CALL Patients in Remission

Case No	BM		Percentage of J5+ Cells in PB	
	Cellularity	Percentage of J5+ Cells	PBL	E−*
1	N	0	2	7
2	N	12	15	15
3	M	6	3	4
4	N	9	3	7
5	N	0	2	2
6	N	9	2	2
7	H	0	2	3
8	M	ND	0	2
9	N	ND	0	0
10	N	5	2	3
11	H†	0	0	1
12	M	0	0	6
13	N	0	0	2
14	H	ND	2	ND
15	H	ND	1	ND
16	N	0	0	3
17	M	1	1	2
18	N	1	2	3
19	N	ND	1	ND
20	N	ND	1	ND

Abbreviations: N, normal, M, median, H, hypoplastic, ND, not determined

*E− cells purified after rosette formation of PBL with AET-SRBC

†Hypoplastic with lymphocytosis.

between the weak staining that we found on these cells and the bright fluorescence of J5+ lymphoid cells.

Colony formation. Normal PB E− cells could generate between 100 and 900 colonies/10^5 plated cells. Cells from the colonies were pooled and characterized for various surface markers. They had no receptor for SRBC, and 65% were sIg+ and 25%, Ia+. In addition, in two subjects tested, 40% and 58% of the colony cells expressed the antigen B1.[14] This phenotype (E−, sIg+, B1+) is characteristic of mature B cells and demonstrates that these culture conditions allow the specific growth of B lineage cells. Morphologic studies confirmed these observations since most of the colony cells had a plasmacytoid appearance as judged after Wright-Giemsa staining. Of note is the fact that none of the cells present in these colonies bore the CALLA antigen, as shown by their lack of reactivity with the J5 antibody.

E− cells from CALL patients in remission were cultured under the same conditions, and the assay was technically satisfactory in 15 patients (cases, 1 to 15). From 20 to 2,400 colonies/10^5 cells were generated As was the case for normal subjects, very few E+ cells were present in the colonies. A total of 57% of these cells were sIg+, which is less than in controls, and 43% were Ia+, significantly more than in normals ($P < .01$). Moreover, in two cases out of two, more than 50% of these cells were stained by the B1 monoclonal antibody (68% and 50%) These results are thus suggestive of a B cell nature for most of these patients' colony cells. A major phenotypic difference, however, was found between the cells derived from CALL patients and those of normal subjects since a mean of 18% of the former expressed CALLA (range, 2% to 72%; Fig 1). The morphology of the patients' colony cells also showed that they were not similar to the controls' cells, since most of them had a lymphoblastoid appearance. Both surface markers and cytologic criteria therefore seemed to indicate that colony cells derived from B precursors are less differentiated in CALL patients in remission than in normal subjects.

To assess whether chemotherapy could affect the differentiation of B cells, five T-ALL and two AML patients in remission and under the same maintenance treatment were tested. From 150 to 500 colonies/10^5 plated cells were generated in T-ALL. A total of 65% of these cells were sIg+ and 28% Ia+, the phenotype being similar to that observed in normal colonies. In all cases, no J5+ cells were detected in the colonies, even in T-ALL patient with circulating J5+ cells (Fig 1).

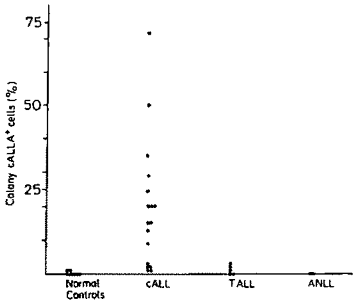

Fig 1. J5-positive cells in pooled colonies generated from the E− cell fraction.

Characterization of B colony-forming cells (B-CFC). An interesting observation was the lack of statistically significant correlation between the proportion of J5+ cells in the colonies of CALL patients and the level of circulating J5+ cells. Therefore, in these patients we studied the differentiation of B-CFC under the same culture conditions but after elimination by complement-mediated lysis of the J5+ cells present in the E− populations. The colonies could be analyzed in 11 patients (cases 1 to 11); lysis of J5+ cells prior to culture did not significantly alter either the plating efficiency (268 ± 145 *v* 354 ± 260 colonies/10^5 cells in the controls, 858 ± 891 *v* 628 ± 526 colonies/10^5 cells in the CALL patients) or the phenotype (E−, sIg+, Ia+) in both controls and CALL patients. A major phenotypic difference, however, was found between the cells derived from CALL patients and those of normal subjects, since a mean of 17% of the former expressed CALLA (range, 2% to 65%). These observations seemed to indicate that in CALL patients, CALLA+ cells can be generated in vitro from CALLA− progenitors.

To further characterize the phenotype of B-CFC, E− cells were treated with anti-Ia antibody (1:600 vol/vol) in addition to J5 (1:800 vol/vol) in two CALL patients and three controls. In both groups this pretreatment led to an abrogation of colony growth (27 ± 6 *v* 104 ± 31 colonies/10^5 cells in the controls, 50 ± 12 *v* 415 ± 49 colonies/10^5 cells in CALL patients), suggesting that the B progenitor cells in normal subjects as well as in the patients bear the Ia antigen. The presence of Ia antigen on normal B-CFC has already been reported by Taetle et al.[24] To assess that the effect of the anti-Ia antibody was not due to a depletion of an accessory cell population, which could be critical to colony growth, increasing numbers of autologous (10^4 to 10^7 cells/mL) adherent cells were added to the E−, J5−, Ia− cell population. The presence of these monocytes could not restore colony growth (unpublished data).

Self-renewal. To investigate the clonogenic capacity of B colony cells from CALL patients and controls, primary colonies were picked individually, pooled, and replated as primary colonies. A significant secondary plating efficiency (PE2) was observed in four of four patients studied. Although considerable variation was present among the patients, essentially no differences were evident between E− and E-J5− secondary plating efficiencies. The proportion of J5+ cells in the secondary colonies was comparable to that found in the primary cultures These results seem to indicate that some colony cells present self-renewal capacity similar to that of stem cells. In contrast, PE2 in controls was very low (inferior to five colonies/10^5 seeded cells) either in E− or in E-J5− fractions.

Cytogenetic analysis. The karyotype of cultured cells was studied in four patients for whom chromosomal abnormalities had been found in the original lymphoblasts (Table 2) During the acute phase, three patients had a mosaic composition of BM lymphoblasts, with 33% of the cells carrying a marker for the first patient, 55% for the second, and 25% for the third patient, the marker being different in each case. Cytogenetic examination of 22 mitoses in the colony cells of the first patient revealed that one cell (ie, 5%)

Table 2. Cytogenetic Analysis of Colony Cells

	BM During Acute Phase				B Colony Cells During Remission		
Case	Lymphoblasts (%)	J5+ cells (%)	Number of Metaphases Studied	Karyotype	J5+ Cells (%)	Number of Metaphases Studied	Karyotype
14	79	85	52	67% 46XX	4	22	95% 46XX
				33% 47XX + mar			5% 47XX + mar
15	90	77	61	45% 46XX	20	20	85% 46XX
				55% 47XX + mar 1			5% 47XX + mar 1
							5% 48XX + mar 1 + mar 2
							5% 47XX + mar 2
19	80	88	45	75% 46XY	24	20	75% 46XY
				25% 53XY +6 +15 +17 +18 +18 +21 + mar			5% 47XY +15
							5% 47XY +mar
							5% 48XY +mar +21
							10% 47XY +21
20	70	75	40	50% 47XY +21	33	20	80% 47XY +21
				50% 46XY +21 −18			5% 46XY +21 −4
							5% 46XY +21 −7
							5% 46XY +21 −18
							5% 46XY +21 −19

had the same marker as the lymphoblasts. In the second patient, out of 20 mitoses studied, one cell had the same marker (mar 1) as the original malignant clone, one cell displayed a new marker (mar 2) and one cell had both markers (mar 1 + mar 2). Thus a total of 15% of the cells had chromosomal aberrations. In the third patient, cytogenetic examination of colony cells revealed a reduction of the hyperploidy observed on 25% of the initial lymphoblasts (from 53 to 47 chromosomes) in 20 mitoses studied; two of them had the same marker as the original malignant clone. The fourth patient had Down's syndrome, presenting a change of the modal chromosome number on 33% of the lymphoblasts at diagnosis (Table 2); out of 20 mitoses studied in the colony cells, one cell shared the loss of the same chromosome (−18), and three cells displayed the loss of three other different chromosomes (Table 2). It is interesting to note that the percentage of J5+ cells in the colonies of these patients was 4%, 20%, 24%, and 33% respectively. In a fifth patient, no chromosome abnormalities were present in the colony cells or in the blasts at presentation (unpublished data).

DISCUSSION

To induce the clonal expansion of circulating B cells in CALL patients in remission, we used an in vitro assay that was found suitable for the generation of B cell colonies from malignant progenitors in CALL,[19] chronic lymphocytic leukemia,[25] B cell non-Hodgkin's lymphoma,[18] and from normal precursors in healthy subjects.[18] B cell colonies derived from normal controls are composed of E−, sIg+, Ia+, B1+ cells that have a plasmacytoid appearance. Patient's colony cells are composed of E−, B1+ (in two cases tested); sIg is expressed on fewer cells than in normals. The Ia determinant, which is lost during the final stages of B cell maturation, is retained by a significantly greater proportion of the patients' colony cells Moreover, a variable fraction of these cells express CALLA, which is associated with immature B cells. In addition, they predominantly have a lymphoblastoid appearance contrasting with the uniform plasmacytoid features of normal colony cells. Taken together, these results suggest that some circulating B cells from CALL patients in remission cannot differentiate in vitro under culture conditions that permit the maturation of normal B cells.

Since it has been shown that chemotherapeutic treatment can induce abnormalities of in vivo lymphopoiesis in leukemic patients,[6,26,27] we have studied T-ALL and AML patients during maintenance therapy. In contrast to CALL, no circulating J5+ cells were detectable in any of these patients except one. In addition, the phenotype and appearance of their B colony cells were identical to those of normal controls. Thus, it seemed unlikely that the chemotherapeutic regimen induced the abnormal in vitro growth pattern of B cells in CALL patients.

A high number of secondary colonies was observed in all four patients for whom primary colony cells were replated, whereas very few secondary colonies could be generated in the controls. Thus, some of the patients' colony cells were capable of self-renewal, a property usually associated with stem cells and malignant cells.

The results of cytogenetics studies in four patients clearly showed that some colony cells were derived from the original malignant clone. In all cases, a fraction of the cultured cells presented a chromosomal aberration identical to that previously found in the initial lymphoblasts. It thus appeared that despite complete hematologic remission these patients had in their PB residual cells belonging to the malignant clone. The expansion of these circulating precursors during the culture allowed the detection of their abnormal progeny by classic cytogenetic methods. All patients are still in remission, two of them more than 18 months after being studied. Since chromosome examination and study of surface markers cannot be done on the same cells, we could not determine the phenotype of the colony cells with chromosomal aberrations. It should be noted that in all cases the percentage of J5+ cells and number of abnormal metaphases in the colonies were comparable. However, further work is needed to establish whether the clonal cells express the J5 determinant. Double staining for J5 and surface or cytoplasmic light chains could be helpful but is subjected to the concomitant expression of CALLA and Ig light chains by the same cell. Such a phenotype has not been described in normal cells and can be induced in vitro in CALL leukemic blasts of only some patients.[16,17] In any case, fluoresceinated anti-κ and anti-λ chains will be used instead of polyvalent anti-F(ab')$_2$ in our subsequent studies to evaluate the distribution of these markers in the whole colony population and eventually in the J5+ fraction.

Since the elimination of CALLA+ cells from the starting population did not alter the phenotype of the cultured cells, it appears that the J5+ colony cells are not, or at least not exclusively, derived from the circulating J5+ cells detected in these patients. The J5+ antibody has been shown to induce the modulation of the CALLA molecule.[28] However, under the technical conditions used in this study (ie, incubation with J5 at 4 °C and subsequent incubation at 37 °C after the addition of complement), lysis is likely to occur prior to any modulation. These results can be compared to the data of Izaguirre and Greaves[29] who showed, by separating CALL blast cells into J5+ and J5− fractions, that CALLA does not seem to be a marker for all potentially clonogenic CALL blasts.

Finally, it is worth mentioning that during the course of this study, three patients (cases 1, 2, 13) relapsed 1, 5, and 2 months, respectively, after being studied. The proportion of J5+ cells in the colonies was 50%, 72%, and 13% respectively. Two of these patients (1, 13) had virtually no BM or PB CALLA+ cells at the time of study. Moreover, no patient in the group of less than 5% CALLA+ colony cells has relapsed thus far. More extensive follow-up might indicate whether a correlation between the percentage of CALLA+ colony cells and subsequent clinical course can be established.

In conclusion, we have shown that some circulating B clonogenic cells of CALL patients in remission do not differentiate in vitro under experimental conditions that allow the maturation of normal B cells. The self-renewal capacity of some of those cells, the presence of an identical karyotypic abnormality in some cultured cells in the original lymphoblasts, and the subsequent relapse of three patients who had a relatively high proportion of J5+ colony cells seem to indicate that this culture system permits the clonal expansion of residual circulating cells linked to the malignant clone. This observation might be of biologic as well as clinical importance, since these residual cells cannot be detected by classic hematologic, cytologic, and even immunologic methods. It would be of interest to study with the same colony assay the differentiation of B cells in CALL patients who are in long-term remission and not receiving therapy

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Application of Hyperthermia to the Treatment of Human Acute Leukemia: Purging Human Leukemic Progenitor Cells by Heat

By Yoshiaki Moriyama, Miwako Narita, Ken Sato, Masaru Urushiyama, Satoru Koyama, Hideo Hirosawa, Kenji Kishi, Masuhiro Takahashi, Kazue Takai, and Akira Shibata

The application of hyperthermia to the treatment of neoplastic disease has focused on solid tumors. Since the hyperthermic sensitivity of human acute leukemia cells is not known, we have studied the in vitro response of human leukemic progenitor cells (L-CFU) to hyperthermia using a quantitative assay system for L-CFU. Human L-CFU were found to be more sensitive than committed normal myeloid progenitor cells to hyperthermic killing (41 to 42 °C). In addition, in the five acute myelogenous leukemic patients studied, it was shown that their leukemic progenitor cells—all types were studied according to the French-American-British diagnosis—were unable to form colonies when exposed to a temperature of 42 °C for 60 minutes, whereas the residual normal clones suppressed by the leukemic cell population were found to recover and to form more colonies in vitro as compared with untreated leukemic marrows. This strongly suggests that in vitro hyperthermia may selectively purge residual leukemic cells, especially L-CFU in stored remission bone marrow before autologous bone marrow transplantation.

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IN THE PAST DECADE, there has been considerable interest in the use of hyperthermia to treat cancer. Elevated temperatures of 41 to 46 °C have been amply demonstrated to inhibit proliferation of malignant cells, which are more sensitive to hyperthermic killing than their normal cell counterparts.[1-3] However, there has been no information on the thermal sensitivity of progenitor cells (L-CFU) from human acute myelogenous leukemic patients (AML), which give rise to colonies consisting of leukemic cells.[4,5] We investigated the effect of hyperthermia on human L-CFU in vitro, using a quantitative human L-CFU assay developed specifically to clone myeloid leukemia by preincubation of leukemic cells with phytohemagglutinin–lymphocyte-conditioned medium (PHA-LCM).[6,7] The results showed that L-CFU in human acute leukemia are more sensitive than committed normal myeloid progenitor cells (CFU-c) to temperatures of 41 and 42 °C. These results suggest that in vitro hyperthermia may selectively eradicate residual leukemic cells in stored bone marrows prior to autologous bone marrow transplantation in humans.

MATERIALS AND METHODS

Leukemic cells Human leukemia cells (57% to 95%) for L-CFU assay were obtained from five patients with AML prior to chemotherapy (Table 1) Normal marrow cells used as controls were obtained from two normal volunteers and from three patients with acute leukemia in complete remission. The marrow cells were aspirated from the sternum or posterior iliac crests using conventional techniques and diluted immediately in McCoy's 5A medium (GIBCO, Grand Island, NY) containing heparin (10 U/mL) The buffy coat was obtained by centrifugation for five minutes at 200 g and washed twice with McCoy's 5A medium. In one patient studied, peripheral blood was obtained by venipuncture into heparinized syringes. Mononuclear cells were then separated by layering the blood over Lymphoprep (Nyegaared, Oslo) and centrifuging for 25 minutes at 400 g Cells in the interface band were harvested and washed twice with McCoy's 5A medium For heating, the cells obtained were adjusted to 2 × 10^6/mL.

Heating Cells suspended in 1 mL of McCoy's 5A medium in Falcon 3033 culture tubes (Falcon Labware, Oxnard, Calif) were immersed in a constant water bath (±0.1 °C) and exposed to temperatures of 41 and 42 °C for 15 to 60 minutes Control cells were kept at room temperature for the same time period After hyperthermic treatment, the cell suspension was immediately cooled in water (4 °C) and prepared for L-CFU and CFU-c assay.

Stem cell assays. Basically, our PHA assay for L-CFU consists of two phases: an initial liquid phase of 15 to 24 hours at 37 °C and a semisolid phase for seven days of incubation[6] Briefly, in the liquid phase, 0.1 mL (for one plate) of McCoy's 5A medium containing 2 × 10^5 marrow or peripheral mononuclear cells was cultured without any serum in a Falcon 3033 culture tube to which 0.05 mL PHA-LCM was added. After preincubation, the cells were not washed, but diluted with 0.9 mL of 0.3% agar medium (enriched McCoy's 5A medium[8] + 15% fetal calf serum) and pipetted carefully to create a single-cell suspension. After being resuspended in an agar medium, the cells were plated into 35-mm Falcon Petri dishes and cultured in a solid phase without any other stimulators.

The CFU-c assay was performed according to the method of Burgess et al[9] using human placenta-conditioned medium (HPCM) as a source of colony-stimulating factor (CSF) to compare to the thermal sensitivity of L-CFU. All cultures were done in duplicate or triplicate

After seven to ten days of incubation in a 5% CO_2 gas-controlled humidified incubator at 37 °C, colonies containing 40 or more cells were counted with an inverted microscope. For determining stem cell survival, the ratio of the colony count in each heated sample to the mean control colony count was calculated.

RESULTS

A comparison between the thermal sensitivity of human L-CFU and CFU-c is illustrated in Fig 1. The survival of leukemic progenitor cells in AML (patient T.O) decreased exponentially with continuous heating at temperatures of 41 and 42 °C. At both temperatures the survival curves for L-CFU had an initial shoulder region. At 42 °C the survival of CFU-c also decreased exponentially, but was not affected at the lower temperature of 41 °C. The terminal slopes of the curves clearly demonstrated greater hyperthermic sensitivity of L-CFU when compared to CFU-c at both temperatures studied. The slope of the exponential portion of the curves for L-CFU increased over ten times with a 1 °C rise in tempera-

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Table 1. Clinical Data and L-CFU Survival of Five Patients With AML by Heat at 42 °C

Patients	Age and Sex	FAB Diagnosis	Hb (g/dL)	Platelets (× 10⁴ μL)	WBC (μL³)	Blasts in Marrow (%)	Survival of L-CFU at 42 °C (No of Colonies)*		
							0 min	30 min	60 min
T.O	51M	M1	6.8	4.1	1600	90 2	182 ± 16	31 ± 4.2	0.3 ± 0.5
Y A.	34F	M2	11.2	5 9	2400	57.5	46 ± 8	4 ± 0.5	0
T.K	65M	M2	5 2	2.4	11000	95 0	202 ± 14	28 ± 5 0	0
							62 ± 8†	4 ± 0 9†	0†
S.I	56F	M4	8.2	0.8	23600	86 2	314 ± 23	18 ± 2 2	0
K K.	38M	M5	6.3	3.6	34800	88 6	102 ± 11	3 ± 0.1	0
Normal CFU-c (n = 5)							116 ± 27	108 ± 15	63 ± 14

Abbreviation: FAB, French-American-British.

*Mean ± SD colonies/2 × 10^5 bone marrow cells

†Assayed for L-CFU of peripheral mononuclear cells (blasts, 75%)

ture, whereas that for CFU-c increased only about 1.8 times.

FAB diagnosis,[10] the clinical outline of five patients with AML, and the thermal sensitivity of L-CFU in each patient at 42 °C are summarized in Table 1. All leukemic progenitor cells (L-CFU) studied, even those producing any type of leukemic cells according to FAB diagnosis, were found to be very sensitive to heat at 42 °C. In addition, L-CFU obtained from four out of five patients were completely killed by heat for 60 minutes at 42 °C and therefore unable to form colonies in vitro, whereas 54% of normal CFU-c survived for the same time period.

To see whether hyperthermic treatment could save normal clone but selectively eradicate leukemic cell populations in leukemic marrows containing normal clone, the possibility that residual normal clones suppressed by the leukemic environment could be detected after purging the leukemic cell population by hyperthermia was examined using a standard agar method.[9] Table 2 is based on the data of an experiment in which four leukemic marrows containing 57.5% to 98.8% blasts were cultured for a CFU-c assay using HPCM as a source of CSF before and after heating for 60 minutes at 42 °C, and three out of these four leukemic marrows were also investigated by an L-CFU assay. The four untreated leukemic marrows obtained from three patients with AML and one with acute lymphocytic leukemia (ALL) grew none or a few CFU-c–derived colonies in an agar medium. After hyperthermic treatment, on the other hand, the L-CFU population disappeared (in three out of four patients, see Table 1), but the residual normal CFU-c in all of the four leukemic marrows studied remained active and appeared to form more colonies in vitro.

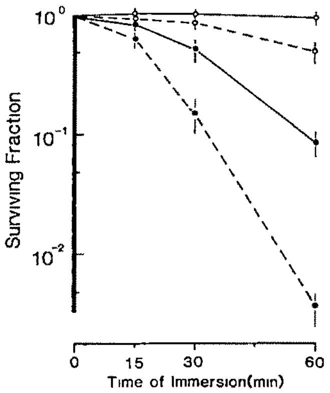

Fig 1. Survival curves for human L-CFU and CFU-c heated for varying lengths of time at different temperatures. Bars, SD; number of colonies at room temperature (controls): O, CFU-c (n = 5), 116 ± 27/2 × 10^5 bone marrow cells; ★, L-CFU (T.O., M1), 182 ± 16/2 × 10^5 bone marrow cells. ——, at 41 °C, ---, at 42 °C.

DISCUSSION

Human acute leukemia is considered to be a clonal hemopathy originating in pluripotential stem cells, and leukemic cells are produced by L-CFU, which are able to self-renew and proliferate in vitro.[7,11,12] Therefore, it is necessary to eradicate the L-CFU population in the bone marrow of patients for a successful treatment of acute leukemia. In humans, attempts have been made to purge malignant cells from marrow using 4HC, ASTA-Z-7557, and monoclonal antibodies.[13-15] Experience is still too limited to evaluate the success of these agents.

In this study, using a quantitative assay system for L-CFU, we clearly demonstrated that the survival of human L-CFU decreases exponentially with continuous heating at temperatures of 41 and 42 °C and that L-CFU in AML are more sensitive than CFU-c to hyperthermic killing. In addition, most of the L-CFU obtained from the marrow or peripheral mononuclear cells of five patients with AML appeared to be unable to form colonies and were eradicated by heat at 42 °C for 60 minutes, whereas over half of the normal CFU-c remained active and survived for the same time period.

Table 2. Survival and Recovery of Residual Normal Clones (CFU-c) in Four Leukemic Marrows Before and After Heating for 60 Minutes at 42 °C

Patients	Age and Sex	FAB Diagnosis	No of CFU-c-Derived Colonies*	
			Before	After
Y.A.†	34F	M2	2 5 ± 0 7	48.0 ± 4 5
T K.†	65M	M2	1 0 ± 0	2.0 ± 1.0
K.K.†	38M	M5	0	3.0 ± 0
H.M ‡	32F	L2	0	3.6 ± 0 6

*Mean ± SD/2 × 10^5 bone marrow cells

†See Table 1

‡Patient with ALL (blasts in bone marrow, 98 8%)

In general, leukemic marrows in humans contain normal clones, but these normal clones are usually suppressed by a leukemic cell population, showing almost no growth of CFU-c–derived colonies in vitro.[16] However, after heating leukemic marrow cells in vitro, residual normal clones (CFU-c) appeared and formed more colonies than untreated marrows, indicating the possibility of purging a leukemic cell population selectively by heat. The loss of the clonogenic capacity of L-CFU with heat is not understood but may be caused by an alteration of cell morphology or perturbation of the cytoskeletal system.[3,17,18] In studies with the AKR murine model, Robins et al[3] demonstrated by electron micrographic studies of AKR leukemia cells treated with 41.8 °C that structural damage to the cell, ie, changes in the Golgi apparatus, was associated with the lack of ability to form colonies and that this decrease in colony formation resulted in increased survival in leukemic AKR mice that received 41.8 °C whole-body hyperthermia. In addition, the electron microscopic studies on the CA755 murine model demonstrated that the Golgi apparatus changes induced by heat correlated with tumor cell death, with an increase in survival, and with cures in some animals.[18] These observations suggest that the lack of the ability of heated leukemic cells to form colonies may represent cell death. Interestingly, however, murine stem cell studies[19,20] also suggested that pluripotential stem cells (CFU-s) are less sensitive than CFU-c to hyperthermic killing because of a division delay of CFU-s, which mostly remain in the G_0 phase.[21] This finding seems to be important for keeping the CFU-s population, which is essential to hematopoietic regeneration after bone marrow transplantation, from being killed while purging residual leukemic cells in stored bone marrows by heat.

From these and our previous results, we emphasize that the in vitro treatment of stored remission bone marrow with hyperthermia may selectively eradicate the L-CFU population before autologous bone marrow transplantation. Thus, in vitro hyperthermia may be a useful tool in clinical bone marrow transplantation for the treatment of human acute leukemia as an alternative to monoclonal antibody treatment for purging leukemic cells in vitro.

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Role of Arginine Residues in the Coagulant Activity of High Molecular Weight Kininogen

By Jin-Jyi Chang, Cheryl F. Scott, and Robert W. Colman

High molecular weight (HMW) kininogen, the cofactor for activation of the contact system of plasma proteolysis, transports and optimally positions prekallikrein and factor XI on a negatively charged surface, allowing those zymogens to be activated by surface-bound factor XIIa. HMW kininogen circulates in plasma as a procofactor that, after cleavage by kallikrein or factor XIIa, gains ability to bind to the surface. The mechanism responsible for this increased affinity for the surface is unknown. We hypothesized that modification of arginine residues may prevent cleavage of HMW kininogen, since the initial kallikrein-induced cleavage sites on the HMW kininogen molecule are at the NH_2 terminal and the COOH terminal of the bradykinin-containing portion of the molecule, each of which contains arginine. We found that modification with butanedione of four arginine residues in the HMW kininogen molecule prevented bradykinin release, which results from cleavage of HMW kininogen. Furthermore, HMW kininogen coagulant activity was lost, in proportion to the degree of arginine modification, until 6.6 residues had been modified. Complex formation with prekallikrein, however, was found to be unaffected by the modification of modified HMW kininogen. To account for the loss of coagulant activity, we also examined the ability of modified HMWKa (active cofactor) to bind to an activating surface. The affinity of modified HMWKa for kaolin was tenfold less than the affinity of unmodified HMWKa. These data suggest that arginine residues play a critical role in the ability of HMW kininogen to function as an activation cofactor, both by preventing the cleavages that produce HMWKa as well as by decreasing the affinity of HMWKa for the surface.

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HIGH MOLECULAR WEIGHT (HMW) kininogen functions as the cofactor of surface-mediated defense reactions[1] by virtue of its ability to position optimally the zymogens, prekallikrein[2] and factor XI,[3] that exist in plasma as biomolecular complexes with HMW kininogen, on the surface where these zymogens can be cleaved by activated factor XII[4–7] Plasma kallikrein cleaves HMW kininogen to disulfide-bridged heavy and light chains, releasing bradykinin in the process. The light chain of HMW kininogen expresses the coagulant activity, since it contains both the surface-binding region as well as the domain for complex formation with either prekallikrein or factor XI.[8]

Recently, we found that HMW kininogen exists as a procofactor, since after proteolysis by kallikrein, the ability of the cleaved protein (HMWKa) to adsorb to a kaolin surface in the presence of plasma concentrations of fibrinogen[9] is markedly enhanced. The molecular changes responsible for this increased affinity for the surface are unknown, since the amino acid residues responsible for the ability of human HMW kininogen to adsorb to surfaces have not been elucidated. In bovine HMW kininogen, it has been suggested that the positively charged residues in a histidine-rich area of the molecule[10] may be critical for binding. We hypothesized that since the primary cleavage sites in the HMW kininogen molecule by plasma kallikrein are on either side of the bradykinin moiety[11] and that since bradykinin contains arginine at both its NH_2 terminal and COOH terminal, modification of arginine residues might prevent cleavage of HMW kininogen by plasma kallikrein. After covalently modifying from 2 to 17 of the 35 arginine residues in HMW kininogen with butanedione, we assessed the effect of these molecular alterations on HMW kininogen by examining coagulant activity, proteolytic cleavage, release of bradykinin, binding to a negatively charged surface, antigenicity, and ability to form a complex with prekallikein.

MATERIALS AND METHODS

Reagents All chemicals used were reagent grade or better Butanedione was purchased from Aldrich Chemicals, Milwaukee. Sodium dodecyl sulfate (SDS), ammonium persulfate, methylene bis-acrylamide, acrylamide, and high and low molecular weight standards (200,000; 116,000; 93,000; 66,500; 45,000; 31,000; 21,000; 14,000) were purchased from Bio-Rad, Richmond, Calif. Dithiothreitol and iodoacetate were obtained from Pierce Chemical Co., Rockford, Ill. Soybean trypsin inhibitor and β-mercaptoethanol were purchased from Sigma Chemical, St Louis. Glass tubes (6 × 50 mm) and kaolin (acid washed) were obtained from Fisher Scientific Co, King of Prussia, Pa. Inosithin was purchased from American Concentrates, New York. Coomassie brilliant blue R-250 was purchased from LKB, Rockville, Md D-Pro-Phe-Arg-*p*-nitroanilide (S-2302) was obtained from Helena Laboratories, Beaumont, Tex Corn trypsin inhibitor was a gift from Dr Edward P. Kirby of the Temple University Health Sciences Center.

Plasma. Normal pooled plasma, used as a reference standard, was purchased from George King, Biomedicals Inc, Overland Park, Kan Prekallikrein-deficient plasma was graciously supplied by Dr Charles Abildgaard, Davis, Calif. Plasma deficient in total kininogen[12] was donated directly to us by Mrs M. Williams, Philadelphia.

Amino acid analysis. Composition of HMW kininogen and the separated polypeptide chains was determined after hydrolysis with 6N HCL at 110 °C for 20, 40, and 60 hours, using the Glenco MM070 micro amino acid analyzer with the Pico System II buffer system (Pierce Chemical Co, Rockford, Ill). The values for serine and threonine were extrapolated to zero time.

Purified plasma proteins. Purified plasma kallikrein was prepared, as previously reported by our laboratory, by activation of purified prekallikrein[13] with factor XII fragments (XIIf) The activation was stopped by inhibiting the XIIf with purified corn trypsin inhibitor (50 μg/mL) The preparation of kallikrein contained no measurable factor XI, XIa, XII, XIIa, plasminogen, or plasmin, as determined by coagulant and amidolytic assays. The preparation, unreduced, showed a doublet (88- and 85-kDa); reduced, it showed a heavy chain of 52-kDa and two light chains of

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36- and 33-kDa. The assay of kallikrein was performed with the amidolytic substrate, S-2302.[13] The specific activity was 55 μmol S-2302 hydrolyzed/min/mg protein.

HMW kininogen was prepared by the method of Kerbiriou and Griffin.[14] On reduced SDS polyacrylamide gel electrophoresis (PAGE), 90% of the molecule was in the form of the intact 120-kDa polypeptide. The specific coagulant activity varied from 15 to 23 U/mg The coagulant activity of the intact molecule or its chains was determined by a modification of the partial thromboplastin time, as previously detailed.[9] The protein concentration of HMW kininogen was determined by measuring UV absorption using $A^{1\%}_{280\ nm}$ = 7 0[14] as well as from amino acid analysis.

Modification of HMW kininogen and cleaved HMW kininogen with butanedione. Modification of arginine residues of HMW kininogen or cleaved HMW kininogen (HMWKa) with butanedione was performed according to the method of Riordan.[15] HMW kininogen (33 μmol/L) was diluted in 50 mmol/L sodium phosphate buffer, pH 7.4, and various amounts of butanedione, diluted 1:100 in the same buffer, were added At various time intervals, aliquots of reaction mixture were pipetted into equal volumes of glacial acetic acid to stop the reaction. HMW kininogen coagulant activity was determined at each time point by pipetting a suitable amount (1 to 2 μL) of the reaction mixture into tubes containing coagulation reagents HMW kininogen with or without acetic acid at time zero had identical coagulant activity. The number of arginine residues modified was determined by difference from amino acid analysis as described above after freeze-drying the acidified aliquots, since the modified arginines are not detected on the chromatograms

Preparation of cleaved HMW kininogen (HMWKa). HMW kininogen, intact or modified with butanedione, was incubated with kallikrein using the conditions described by Scott et al.[9] In brief, HMW kininogen in .015 mol/L Tris-acetate, pH 8.0, was incubated with kallikrein (molar ratio of 1.100) at 37 °C. At specific times, aliquots were pipetted into microfuge tubes containing a tenfold molar excess of soybean trypsin inhibitor to inhibit kallikrein and stop the cleavage process. A portion of the aliquot was reduced with dithiothreitol (DTT) (1 mmol/L) and subjected to SDS-PAGE according to the method of Laemmli,[16] and the remainder was used for determining the release of bradykinin. Since the cleavage of the two bonds liberating bradykinin are virtually simultaneous, HMWKa is defined for this study as kinin-free HMW kininogen.

Measurement of bradykinin release. Bradykinin was measured using a radioimmunoassay according to the procedure of Proud et al[17] as previously reported.[9] The antibody against bradykinin was a generous gift of Dr David Proud (Baltimore, Md).

Radiolabeling of HMW kininogen. ^{125}I-HMW kininogen was prepared by radiolabeling purified HMW kininogen by the Iodogen method[18] as previously reported.[9] The specific radioactivity was 1 5 × 10^6 cpm/μg. This process did not alter the coagulant activity of the HMW kininogen. The ^{125}I-HMW kininogen was cleaved to ^{125}I-HMWKa as described above. In some cases, the ^{125}I-HMW kininogen was modified with butanedione prior to incubation with kallikrein.

Binding of ^{125}I-HMWKa and butanedione-modified ^{125}I-HMWKa to kaolin. The binding of ^{125}I-HMWKa to kaolin or butanedione-modified ^{125}I-HMWKa was performed according to the procedure of Scott et al.[9]

Antiserum against HMW kininogen. Antiserum against HMW kininogen was raised by immunizing rabbits with HMW kininogen that had been emulsified with complete Freund's adjuvant. Pooled antiserum was rendered monospecific by successive absorption with plasma deficient in HMW kininogen. Purified antiserum showed one precipitin line against HMW kininogen in normal plasma and no reaction with HMW kininogen-deficient plasma by either immunodiffusion[19] or immunoelectrophoresis.[20]

Measurement of prekallikrein–HMW kininogen complexes When prekallikrein is electrophoresed into agarose containing anti-kallikrein antibody, using the method of Laurell,[21] it fails to migrate at pH 8.6, since it is at or near its isolectric point.[22] On addition of HMW kininogen to the purified prekallikrein, "rockets" of successively increasing height are observed, representing prekallikrein–HMW kininogen complexes of anionic mobility. The height of the rocket is proportional to the concentration of HMW kininogen added.[22]

RESULTS

Time and concentration dependencies of modification of HMW kininogen. Butanedione reacts with the guanido groups of arginine and does not affect the ε-amino group of lysine.[15] When HMW kininogen was modified with butanedione, progressive loss of coagulant activity resulted The loss of coagulant activity at various concentrations of butanedione, as expected, followed pseudo-first-order kinetics when performed at excess reagent concentration (Fig 1). The pseudo-first-order reaction rate constant was proportional to butanedione concentration (Fig 1, insert). The calculated second-order rate constant (k) was 3 × 10^6 (mol/L)$^{-1}$ min^{-1}. When butanedione was immediately mixed with HMW kininogen prior to assay, no effect on the coagulant activity was observed, indicating that butanedione itself was not inhibiting the coagulation assay.

The total number of arginine residues in HMW kininogen is 35/mol,[11] representing 3.5% of the total amino acid residues. The number of arginine residues modified and the percentage of coagulant activity remaining are presented in Table 1. For convenience, four species of modified proteins are distinguished. The coagulant activity of butanedione-modified HMW kininogen decreased about 25% when 2 to 3 arginine residues were modified [BD-HMWK (I)]. When one additional arginine residue was modified, the coagulant activity decreased another 15% [BD-HMWK (II)] Further modification of 2 to 3 arginine residues decreased the coagulant activity to about 40% of the original level [BD-

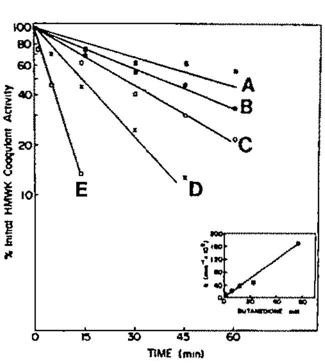

Fig 1. Effect of butanedione modification on HMW kininogen coagulant activity. Purified HMW kininogen was incubated with various concentrations of butanedione. At various times, a portion was assayed for HMW kininogen coagulant activity. The insert shows the concentration dependence of the pseudo-first-order reaction rate from which the second-order rate constant can be calculated. Butanedione concentrations: A, 1.15 mmol/L, B, 5.75 mmol/L; C, 11.5 mmol/L; D, 23 mmol/L; E, 57.5 mmol/L.

Table 1. Effect of Butanedione Modification on the Coagulant Activity of HMW Kininogen

Sample	Arginine Residues Modified*	Specific Coagulant Activity (U/mg)	Percentage of Original Activity
HMW kininogen	0	23.0	100
BD-HMWK (I)†	2.7	17.0	74
BD-HMWK (II)	4.1	14.0	61
BD-HMWK (III)	6.6	8.70	38
BD-HMWK (IV)	17.1	.91	4

*Determined by loss of arginine on amino acid analysis.

†Four levels of modified HMW kininogen were selected, designated BD-HMWK (I)–(IV).

HMWK (III)]. The loss of coagulant activity was directly proportional to the number of arginines modified, up to at least 6.6 residues (Table 1). When almost all of the coagulant activity was lost, about half of the total 35 arginine residues had been modified [BD-HMWK (IV)].

Effect of butanedione modification of HMW kininogen on cleavage by kallikrein. The release of bradykinin by plasma kallikrein involves cleavage of HMW kininogen at lysine-arginine and arginine-serine bonds.[23] Thus it seemed that modification of arginine might affect the cleavage, as assessed by the rate of bradykinin release.

The release of bradykinin from unmodified HMW kininogen by plasma kallikrein gave similar results to those previously reported by Scott et al[9] (Fig 2). Bradykinin was rapidly released from the HMW kininogen by plasma kallikrein during the first 60 minutes and was complete by 120 minutes. After modification with butanedione, however, bradykinin was released at a slower rate. When 2.7 arginine residues were modified [BD-HMWK (I)], bradykinin was slowly liberated, reaching 50% of the maximum level at 60 minutes and 80% after 240 minutes of incubation. With more than 4 arginine residues modified, bradykinin was released from HMW kininogen at an even slower rate and reached only 30% [BD-HMWK (II)] and 15% [BD-HMWK (III) and (IV)] of the control value after 240 minutes of incubation. These results were confirmed by SDS-PAGE analysis of kallikrein digestion of HMW kininogen (120-kDa) to the 65-kDa heavy chains and the 56-kDa and 45-kDa light chains.[9] Although the intact 120-kDa molecule was completely cleaved by 15 minutes in unmodified HMW kininogen, the digestion was slower and traces of the intact molecule persisted at 60 minutes in BD-HMWK (I). In the remaining three modified forms with 4.1, 6.6, and 17 arginines modified, virtually no cleavage of the 120-kDa parent molecule was evident (data not shown).

Butanedione modification of kallikrein-cleaved HMW kininogen. When HMW kininogen was cleaved with plasma kallikrein (HMWKa) prior to incubation with butanedione, it lost coagulant activity at a slower rate than that observed with the intact molecule. The time course of modification of both intact HMW kininogen and HMWKa at two different butanedione concentrations (11.5 mmol/L and 57.5 mmol/L) is shown in Fig 3. HMWKa was more resistant to the butanedione-induced loss of coagulant activity than the intact HMW kininogen. Five times more butanedione was required to inactivate HMWKa than to inactivate the intact procofactor, HMW kininogen. The second-order inactivation rate constant was calculated to be 5.2×10^5 $(\text{mmol/L})^{-1}$ min^{-1} for HMWKa, as compared to the value of 3×10^6 $(\text{mmol/L})^{-1}$ min^{-1} for intact HMW kininogen. Although the rate of modification of HMWKa by butanedione (as reflected by the loss of coagulant activity) was decreased, the extent of modification was not affected. Since HMWKa was modified with butanedione over extended time periods, amino acid analysis showed that 15 arginine residues had been modified. Since bradykinin, which contains 2 arginine residues, was no longer attached to HMWKa, this value agrees with that of 17 arginine residues modified in BD-HMWK (IV).

Binding of butanedione-modified HMWKa to kaolin. The role of arginine residues in the binding of HMW kininogen to a negatively charged surface was studied by

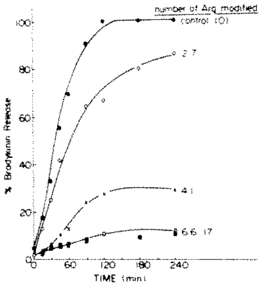

Fig 2. Effect of butanedione on kallikrein-induced bradykinin release from HMW kininogen. HMW kininogen was incubated with butanedione, and the number of arginine residues modified was determined by amino acid analysis (see Methods). HMW kininogen (40 μg), either unmodified or modified to different extents, was incubated with plasma kallikrein (1.4 μg) in a final volume of 300 μL. At various times, a 10-μL portion was transferred to a tube containing 1 μg SBTI. Bradykinin release was determined on each sample.[17] The data are expressed as percent of maximal bradykinin released. Arginine residues modified: ●, unmodified; ○, 2.7 x, 4.1; □, 6.6; ■, 17.

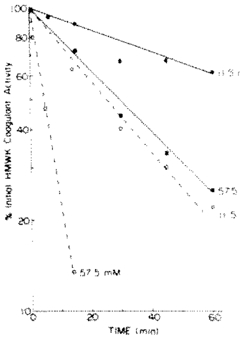

Fig 3. Effect of butanedione on the coagulant activity of cleaved and uncleaved HMW kininogen. Procofactor (HMW kininogen, ---) or active cofactor (HMWKa, ——) was incubated with either 11.5-mmol/L butanedione (●, ○) or 57.5-mmol/L butanedione (■, □). At various times, a portion was assayed for HMW kininogen coagulant activity. The data are expressed as percent of initial HMW kininogen coagulant activity on a semilogarithmic plot.

comparing the binding of unmodified and modified ^{125}I-HMWKa to kaolin. Since intact HMW kininogen binds poorly, if at all, to kaolin[9] and modification with butanedione prevents cleavage, HMW kininogen was cleaved with plasma kallikrein prior to incubation with butanedione. ^{125}I-BD-HMWKa (IV), with 15 arginine residues modified, reached the same saturation point as unmodified ^{125}I-HMWKa (Fig 4). However, about ten times more BD-HMWKa (IV) was needed to achieve the same degree of binding, as compared to the unmodified HMWKa.

Immunochemical analysis of butanedione-modified HMW kininogen. In order to determine whether arginine residues were located in the immunogenic sites of HMW kininogen, antiserum against native HMW kininogen was employed. The results of double diffusion analysis are shown in Fig 5. All modified HMW kininogen samples showed lines of complete identity with unmodified HMW kininogen. These results indicated that arginine residues in HMW kininogen do not critically influence antigen-antibody interaction.

Effect of butanedione modification of HMW kininogen on its complex-formation ability with prekallikrein. HMW kininogen was incubated with butanedione until 90% of the initial coagulant activity was destroyed. This material, or unmodified HMW kininogen, at various concentrations, was mixed with a fixed amount of prekallikrein. These samples were then subjected to electroimmunodiffusion.[22] Both the modified and unmodified HMW kininogen samples were able to increase the height of prekallikrein "rockets" to the same degree as a function of concentration (data not shown). Since this assay is a direct measure of ability to form a complex with prekallikrein,[22] this observation indicates that the loss of coagulant activity of HMW kininogen after modification by butanedione was not the result of the loss of the ability of HMW kininogen to form a complex with the contact system zymogen.

DISCUSSION

Modification of arginine residues of HMW kininogen decreased its coagulant activity (Fig 1) similar to previous observations of two other contact system proteins.[23] By increasing the concentration of butanedione used or by prolonging the reaction time, the coagulant activity of HMW kininogen could be destroyed completely (Fig 1 and Table 1). At least three potential functions of arginines in HMW kininogen may contribute to coagulant activity. First, the positively charged arginine residues may be required for binding to a negatively charged activating surface, and thus their destruction would directly impair the coagulant activity. While this effect is important, this chemical modification alters more than just surface binding. When HMW kininogen was first cleaved by kallikrein to HMWKa and then modified with butanedione, the loss of coagulant activity decreased about fivefold, as judged from the decrease of the pseudo-first-order reaction rate constant (Fig 3). If arginine residues were the only amino acid residues responsible for binding, the cleavage by kallikrein might further expose these residues and the modification with butanedione would have destroyed the activity more quickly rather than more slowly. A second possibility to be considered is that modification of arginine residues impaired the ability of HMW kininogen to form a complex with prekallikrein, since such complex formation is essential for proper alignment of prekallikrein on a negatively charged surface for subsequent cleavage by factor XIIa. However, no impairment of complex formation was found on electroimmunodiffusion. A third possibility is that the loss of coagulant activity is due to the prevention of the cleavage of HMW kininogen by kallikrein, which is necessary for binding to a negatively charged surface.[9] The 2 arginine residues in the nonapeptide bradykinin were apparently modified during the early stage

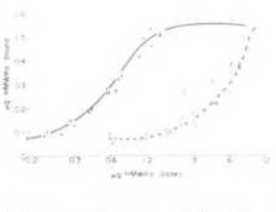

Fig 4. Effect of butanedione modification on the binding of ^{125}I-HMWKa to kaolin. ^{125}I-HMWKa was added to nonlabeled HMWKa. An aliquot was modified with butanedione. Various amounts of either the unmodified (x) or modified (O) HMWKa mixtures were added to microcentrifuge tubes containing 100 μg of kaolin, 300 μg of human serum albumin, and 300 μg of purified fibrinogen in a final volume of 100 μL. After 5 minutes at 25 °C, the samples were centrifuged and the supernatants and pellets were counted for radioactivity.

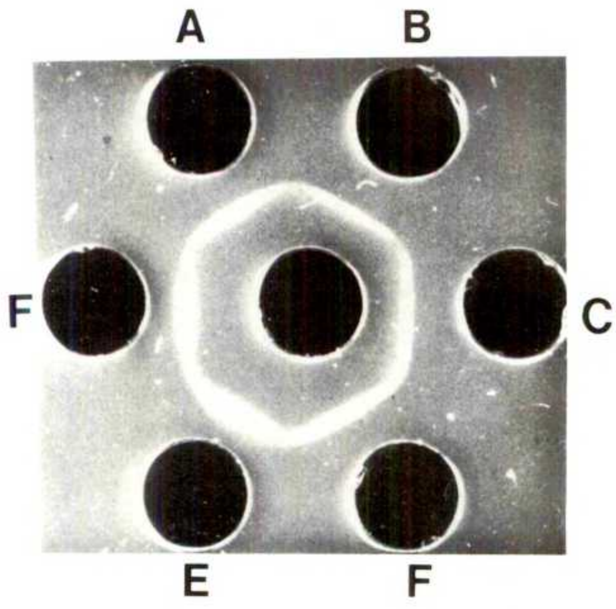

Fig 5. Double immunodiffusion of butanedione-modified and unmodified HMW kininogen. Immunodiffusion was performed in 1% agarose, pH 8.6. The center well contained 20 μL of monospecific antiserum to HMW kininogen. A, D, HMW kininogen unmodified; B, 68% coagulant activity remaining; C, 41% coagulant activity remaining; E, 31% coagulant activity remaining; F, 24% coagulant activity remaining. After the precipitin lines were formed (after 48 hours), the agarose gel was soaked in 0.1 mol/L sodium phosphate, pH 7.0, containing 0.5 mol/L NaCl in order to remove unbound protein. After drying the gel, the protein bands were stained with Coomassie brilliant blue R-250.

of reaction with the protein. Evidence for the modification of these 2 arginine residues was the prevention of cleavage of modified HMW kininogen and the decrease in the release of bradykinin upon incubation with plasma kallikrein (Fig 2). With 2.7 arginine residues modified, plasma kallikrein could still cleave the modified HMW kininogen and release bradykinin from the molecule, only at a slower rate than from an unmodified molecule. However, when more than 4.1 arginine residues were modified [BD-HMWK (II) through (IV)], the cleavage by plasma kallikrein was almost completely prevented, as assessed by SDS-PAGE. Furthermore, bradykinin release was drastically reduced as well (Fig 2). These results suggested that the arginine residue in the lysine-arginine bond in HMW kininogen and the arginine residue of the arginine-serine bond, which must both be cleaved to release bradykinin, are most likely the third and fourth arginines modified. In human HMW kininogen, the arginine-serine bond of bradykinin is first cleaved by kallikrein to generate the 65-kDa heavy chain and the 56-kDa light chain. Further cleavage of the lysine-arginine bond in the COOH terminal of the 65-kDa heavy chain releases bradykinin.[11] The results of butanedione modification also suggested that these 2 arginine residues were located on the surface of the HMW kininogen molecule, as would be expected.

Binding of butanedione-modified HMW kininogen to a model negatively charged surface, kaolin, showed that modification of arginine residues affected the affinity of the molecule to bind to kaolin but not the number of molecules bound (Fig 4). For example, at 1.2 μg HMWKa added, tenfold less modified HMWKa (.05 μg) was observed to bind to kaolin, compared to the binding of unmodified HMWKa (.5 μg). However, the modified HMWKa reached the same extent of binding (.5 μg), albeit at higher amounts added (12 μg) than the unmodified HMWKa. These results suggest that arginine residues influence the affinity of HMW kininogen for negatively charged surfaces, such as kaolin, although they are not absolutely required for binding.

Immunoprecipitation experiments showed that modification of arginine residues did not affect the immunochemical properties of the HMW kininogen molecule (Fig 5). All four modified HMW kininogens, BD-HMWK (I) through (IV), showed lines of complete identity with unmodified HMW kininogen These results indicate that the arginine residues modified are not located in the immunogenic regions of the HMW kininogen molecule.

Thus the mechanism responsible for the inhibition of coagulant activity of HMW kininogen, after modification of arginine groups, appears to be the result of prevention of cleavage of the intact HMW kininogen molecule, which is required to convert it from a procofactor to an active cofactor,[9] as well as a decreased affinity of the active cofactor for the surface.

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Mixed Hematologic Chimerism After Allogeneic Marrow Transplantation for Severe Aplastic Anemia Is Associated With a Higher Risk of Graft Rejection and a Lessened Incidence of Acute Graft-Versus-Host Disease

By Roger S. Hill, Finn Bo Petersen, Rainer Storb, Frederick R. Appelbaum, Kris Doney, Steven Dahlberg, Robert Ramberg, and E. Donnall Thomas

Ninety-six patients with severe aplastic anemia who received a sex-mismatched, HLA-identical allogeneic sibling marrow transplant had sequential cytogenetic analyses performed to determine the incidence and implications of posttransplant mixed hematologic chimerism. Of the 96 patients, 56 (58.3%) became mixed chimeras with coexisting host and donor cells detected in peripheral blood or marrow 14 days or later after transplant, and 40 patients (41.7%) were complete chimeras with 100% donor-type hematopoietic cells. The incidence of mixed chimerism was independent of prior blood production transfusions and infusion of donor buffy coat. The rejection rate was significantly increased in the mixed chimeric group, particularly in patients not receiving buffy coat (14 of 36 rejecting), although overall, the majority (69.7%) retained their first graft. Rejection was seen almost exclusively in patients exposed to multiple transfusions before transplantation. If patients who reject their first graft are censored, the overall incidence of grades II through IV acute graft-v-host disease (GVHD) was significantly reduced in those with mixed chimerism. Transfused patients with mixed chimerism in particular were less likely to develop grades II through IV acute GVHD. The incidence of chronic GVHD was similar in the two groups and did not significantly influence survival. In this study, mixed chimerism persisted for up to 395 days posttransplant, either the first graft being rejected or, more commonly, hematopoiesis reverting to 100% donor-type cells. Mixed lymphohematopoietic chimerism may persist in patients with aplastic anemia who have received matched allogeneic marrow transplants for significant periods before hematopoiesis reverts to donor cell type.

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STUDIES OF BLOOD genetic markers including karyotyping, immunoglobulin allotypes, red and white blood cell enzymes, restriction enzyme fragment-length polymorphism, and red blood cell antigens[1-4] are useful after allogeneic marrow transplantation in establishing the presence of donor- or host-type cells in recipients and thus evaluating the outcome of engraftment. Cytogenetic studies of cells from blood and marrow after sex-mismatched, HLA-matched sibling allogeneic transplantation have proved particularly revealing in patients with aplastic anemia. Disappearance of donor-type and reappearance of host-type cells following allogeneic marrow transplantation for severe aplastic anemia have been associated with marrow graft rejection and, most commonly, the return of the original disease or, rarely, complete recovery of autologous marrow function.[5-14] Even more rarely, donor and recipient cells may coexist (mixed chimerism) for many months.[15] However, the overall frequency of mixed chimerism following allografting for severe aplastic anemia and the implications of mixed chimerism for the outcome of the procedure remain to be established.

In the present studies, we have reviewed sequential cytogenetic analyses of marrow and peripheral blood cells in 96 patients with aplastic anemia given marrow transplants from sex-mismatched, HLA-identical siblings after cyclophosphamide conditioning. The frequency and stability of mixed chimerism have been assessed, and the clinical implications—graft rejection, autologous recovery, or sustained allogeneic engraftment—determined.

MATERIALS AND METHODS

Between July 1970 and June 1984, 244 patients were transplanted for severe aplastic anemia. Of them, 125 (51.2%) were sex-mismatched. Nine of the 125 patients were excluded from analysis because of lack of cytogenetic data, including 4 patients who died within 14 days of transplantation before postgraft cytogenetic analyses were performed Two of the remaining 112 patients had HLA-mismatched transplants, and a further 14 received total body irradiation (TBI) as part of the initial preparatory regimen and were also excluded. A total of 96 patients conditioned with cyclophosphamide, 50 mg/kg IV on each of four successive days, and given HLA-identical sibling transplants were therefore eligible for study. All patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent. Tables 1 and 2 show patient characteristics.

The HLA identities of donor and recipient were determined by serologic histocompatibility typing and mixed leukocyte culture (MLC).[5,7,16] The response in MLC of the patient's cells to sibling cells was compared to their response to pooled freshly drawn cells from two unrelated donors or cryopreserved cells from three unrelated donors and then expressed as a ratio, the relative response index, RRI.[17,18] On the basis of data from 212 healthy HLA-identical siblings, a positive relative response was defined as >2.6% and a negative one was <2 6%. The average relative response in this control was 0.0% (two standard deviations = 2 6%) A positive relative response was interpreted to be the expression of transfusion-induced sensitization of the patient against the donor marrow Details of the methods of assessing allogeneic engraftment by cytogenetic analysis and quinacrine dihydrochloride banding of marrow and peripheral blood cells have been described [1,19] Hematopoietic cells for cytogenetic analysis were collected from either

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Table 1 Clinical Characteristics of Aplastic Anemia Patients

	Mixed Chimera	Complete Chimera	*P* (Test)*
No. of patients	56	40	
Male/female	33/23	25/15	
Patient age (years)†	21 (2–53)	18.5 (3–53)	
Donor age (years)†	19.5 (1–57)	22.5 (7–49)	
Pretransplant transfusions			
No. of patients given RBCs	45	31	
No. of RBC transfusions per patient†	6	6	NS (*t*)
No. of patients given platelets	45	30	
No. of platelet transfusions per patient†	22	29	NS (*t*)
No. of patients untransfused	11	9	
RRI (No. of patients)			
Negative	40	30	NS (χ)
Positive	13	3	
Median bone marrow cell dose			
First transplant†‡	3.10 (.74–16.40) × 10^8	2.89 (1.00–28.20) × 10^8	NS (*t*)

*NS, not significant, *t*, Student's *t* test, χ chi-square test.

†Median (range)

‡Marrow nucleated cells/kg recipient body weight

marrow aspirated at 14, 21, 28, 56, and 80 to 100 days posttransplant or from phytohemagglutinin (PHA)-stimulated blood. Details of the immuosuppressive conditioning regimen for transplantation, marrow infusion, and supportive care for grafting have all been previously described.[5,7,8,16,20]

Two groups of patients were identified. The first group, designated "mixed chimeras," consisted of 56 patients in whom cytogenetically proven host and donor hematologic cells coexisted in either bone marrow or peripheral blood at 14 days or later after the first transplant. A second group of 40 patients, designated "complete chimeras," were comparable patients (Table 1) in whom exclusively donor hematopoietic cells were detected posttransplant.

Statistical analysis. Estimation of survival and the probability of developing grades II throught IV acute graft-*v*-host-disease (GVHD) were calculated using the Kaplan-Meier method.[21] Cox regression models[22-24] incorporating time-dependent covariates indicating chimeric status at each time after transplant were used to examine possible associations of chimeric status with survival and acute GVHD incidence. The score test statistic from the Cox model was used to test the hypothesis of no difference between the mixed

Table 2 Clinical Outcome for Marrow Transplantation Patients

	Mixed Chimera	Complete Chimera	*P* (Test)*
No. of patients	56	40	
No. surviving	33	25	
Survival (days)†	1,904 (85–4,021)	2,175 (197–4,522)	
No. dead (as of July 84)	23 (41.1%)	15 (37.5%)	
Day of death†	72 (24–1,462)	82 (8–714)	
No. rejecting first graft	17	2	.01 (χ)
Patients with sustained engraftment	39	38	
No. receiving buffy coat	17	12	NS (χ)
No. with no acute GVHD	30	14	
No. with acute GVHD	9 (23.1%)	24 (63.2%)	
Grade I	2	4	
II	3	7	.03 (C)
III	2	9	
IV	2	4	
Transfused patients	29	29	
No. with no acute GVHD	24	9	
No. with acute GVHD	5 (17.2%)	20 (69.0%)	
Grade I	2	3	
II	1	6	.002 (C)
III	1	8	
IV	1	3	
No. with chronic GVHD	15	18	NS (C)
No. surviving			
With buffy coat	6/9	6/9	
Without buffy coat	4/6	6/9	

*NS, not significant ($P >$ .05); χ, chi-square test, C, Cox model score test statistic adjusting for time of determination of mixed chimeric status.

†Median (range)

and complete chimeric patients Chi-square and two sample t tests were used to compare chimeric status and clinical characteristics of patients. All significance values were derived from two-sided tests

RESULTS

Of 96 evaluable patients, 56 (58.3%) with severe aplastic anemia who received an HLA-matched, sex-mismatched marrow transplant became mixed hematologic chimeras with host and donor cells coexisting for some period of time after transplant. Forty (41.7%) were complete chimeras with only donor cells present. The median time for detection of mixed chimerism was 18 days posttransplant (range, 14 to 68 days). Initial detection was made from phytohemagglutinin (PHA)–stimulated blood in 36 patients and from marrow in 20 patients. Thirty-five of 56 mixed chimeric patients (62.5%) had 100% donor metaphases by median day 54 (range, 17 to 395 days). The average number of cytogenetic studies performed on each patient between days 14 and 54 was 5.5 (range, 2 to 12), each study representing a different time point posttransplant. Table 1 shows some clinical characteristics of the 56 mixed chimeric and 40 complete chimeric patients analyzed. The respective male-to-female ratio of 33:23 and 25.15, patient median and age ranges of 21 (2 to 53) years and 18 (3 to 53) years, and donor age and pregraft transfusion histories were comparable for the mixed and complete chimeric groups (Table 1). Eleven mixed chimeric and 9 complete chimeric patients received no transfusions of blood products pretransplant. One of 20 untransfused patients rejected the graft and underwent autologous recovery.

The marrow nucleated cell dose received for the first transplant by 56 patients who subsequently developed mixed chimeras was 4.10 × 10^8/kg recipient body weight and 2.89 × 10^8/kg for 40 patients with complete chimerism (not significantly different).

The relationship between prior blood product transfusion and subsequent development of mixed chimeras was evaluated by analysis of the results of RRI for 40 mixed and 30 complete chimeras. Thirteen mixed and three complete chimeric patients had a positive RRI, not significantly different.

Graft rejection. Seventeen patients with mixed chimerism (30.4%) and two with complete chimerism (5.0%) rejected their marrow grafts (Table 2). This difference in rejection rates between the groups is significant (P = .01). Two mixed chimeric patients who rejected their first marrow underwent complete autologous bone marrow recovery.

Patient survival. The clinical outcome is summarized in Table 2. Twenty-three mixed chimeras (41.1%) and 15 complete chimeras (37.5%) died following transplantation. The median day to death in the mixed chimeric group was 72 (range, 39 to 726) and among the complete chimeras, 82 (range, 8 to 714). More than 50% of the 33 surviving mixed chimeric patients have been followed for more than 5.2 years after transplant, and more than 50% of the 25 surviving complete chimeras have been followed for over 5.9 years. The probability of survival for all patients, including those who rejected their first graft, is presented in Fig 1. No significant differences were found between mixed and complete chimeric patients when using a Cox model to adjust for the time at which mixed chimeric status was determined. The Kaplan-Meier estimate of 5-year survival was 62% for complete and 55% for mixed chimeric patients. Figure 2 shows a similar set of curves when patients who rejected their first grafts were excluded from the analyses. The projected survival advantage for the mixed chimeric group is not, however, significant after adjusting for the time of determination of mixed chimeric status (P = .4). The estimated 5-year survival of patients who sustained their first graft was 62% and 70% for the complete and mixed chimeric groups, respectively, with no deaths occurring past 4 years posttransplant.

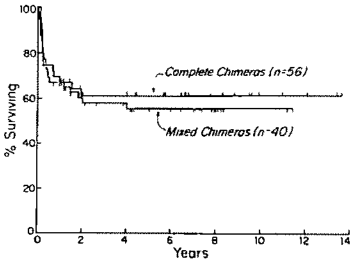

Fig 1. Kaplan-Meier estimates of survival in mixed chimeric and complete chimeric patients, including patients who rejected their first graft. The estimated 5-year survival was 62% for complete and 55% for mixed chimeric patients, not significantly different.

Graft-Versus-Host Disease (GVHD). The incidence and severity of acute GVHD is shown in Table 2. A total of 77 patients—39 mixed and 38 complete chimeras who achieved durable engraftment without rejection—were evaluable for acute GVHD. In the mixed chimeric group, 30 patients had no acute GVHD and 9 developed acute GVHD, including 2 with grade I and 7 with grades II through IV GVHD. Fourteen complete chimeric patients had no acute GVHD and 24 developed acute GVHD, including 4 with grades I and 20 with grades II through IV. The difference in the incidence of acute GVHD grades II through IV between the two groups was significant (P = .03), using the score test from the Cox model. When patients who were tranfused before transplantation were analyzed separately, 3 of 29 mixed chimeras but 17 of 29 complete chimeras developed grades II through IV acute GVHD. This difference in incidence between the groups is highly significant (P =

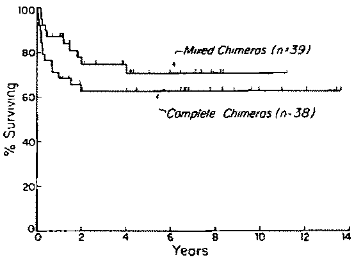

Fig 2. Kaplan-Meier estimates of survival in mixed chimeric and complete chimeric patients, excluding patients who rejected their first graft. The estimated 5-year survival was 62% for complete and 70% for mixed chimeric patients, not significantly different.

.002). The small numbers of untransfused patients (Table 1) precluded a similar acute GVHD analysis The Kaplan-Meier estimate of the probability of developing grades II through IV acute GVHD for mixed chimeric and complete chimeric groups is depicted in Fig 3. The curves are significantly different, showing an increase in the frequency of acute GVHD in the complete chimeric group. By 2 months posttransplant, the incidence of grades II through IV acute GVHD is projected as 18% for the mixed chimeric group and 57% for the complete chimeric groups ($P = .03$, adjusting for the time of determination of mixed chimeric status). Four of 7 mixed chimeras with grade II through IV acute GVHD are surviving, compared with 8 of 20 complete chimeras.

Chronic GVHD. The incidence of chronic GVHD in patients censored for rejection was 15 of 39 in the mixed chimeric group and 18 of 38 in the complete chimeric group (not significantly different; Table 2). Comparable numbers of patients, 9 of 15 and 9 of 18 in the mixed and complete chimeric groups with chronic GVHD, had previously received buffy coat. Ten of 15 mixed chimeric patients with chronic GVHD, including 6 of 9 who received buffy coat and 4 of 6 who received no buffy coat, are survivors. By comparison, 12 of 18 complete chimeric patients with chronic GVHD are surviving, including 6 of 9 who received buffy coat and 6 of 9 who did not. The incidence of chronic GVHD did not significantly influence the survival in these groups.

Donor buffy coat infusion. Twenty of 56 patients (35.7%) with mixed chimerism and 12 of 40 (30%) with complete chimerism had received viable donor buffy coat infusions following transplantation. The effect of receiving buffy coat on graft rejection was evaluated. Three of 20 (15%) mixed chimeras who received buffy coat rejected their grafts and 2 are surviving, both following a second allogeneic graft. Fourteen of 36 (38.9%) mixed chimeric patients who received no buffy coat rejected their grafts and 2 are surviving, both following autologous hematologic recovery. By comparison, 0 of 12 complete chimeras who received buffy coat and 2 of 28 (7.6%) complete chimeric patients who received no buffy coat rejected their grafts. Using a two-sided Fisher's exact test, the difference in rejection rates between mixed and complete chimeric patients not receiving buffy coat is significant ($P = .008$), as is the overall difference in incidence of rejection in the mixed and complete chimeric patient groups.

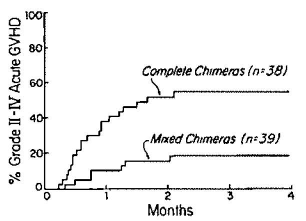

Fig 3. Probability of developing Grades II through IV acute graft-*v*-host disease in complete and mixed chimeric patients. The incidence at 2 months posttransplant is projected as 18% for mixed chimeric and 57% for complete chimeric groups. Significance ($P = .03$) was obtained from a Cox regression model incorporating the time of determination of mixed chimeric status

DISCUSSION

In this study we have demonstrated that a majority (58.3%) of patients transplanted for severe aplastic anemia had evidence of coexisting host and donor hematopoietic cells (mixed chimerism) 14 days or longer after transplantation. The presence of mixed chimerism appeared to be independent of whether or not patients had multiple transfusions before transplant and independent of the use of viable donor buffy coat infusions to ensure engraftment. Patients who were mixed chimeras had a significantly higher rate of graft rejection, 17 of 56 (30.4%), than patients who were complete chimeras, 2 of 40 (5.0%). As a rule, rejection was seen in patients exposed to multiple transfusions before transplantation, suggesting that it was mediated by specifically sensitized host immune cells. Most mixed chimeras (69.2%) did not reject their grafts, and host cells tended to disappear by a median time of 54 days with occasional persistence beyond 1 year. No patient remained a mixed chimera beyond 395 days; by that time patients had either rejected their first graft or converted to complete chimerism.

Only one patient has been reported in the literature to have established long-term stable mixed chimerism after matched allogeneic marrow grafting for aplastic anemia.[15] Our findings suggest that as a rule, surviving host-cells in mixed chimeras are ultimately replaced either as a result of immunologic or competitive removal by donor cells or because host pluripotent stem cells generally lack sustained proliferative capacity to provide durable hematopoiesis, a feature of the original aplastic anemia. This lack of stable mixed chimerism in patients with severe aplastic anemia is distinct from that of dog and rhesus monkey models. Previous animal studies have shown that cyclophosphamide[25-27] and dimethyl myleran[28] can be used instead of total body irradiation (TBI) to prepare recipients for successful marrow engraftment. These chemotherapy chimeras, in contrast to radiation chimeras, rather commonly show a persistent mixture of host and donor lymphopoietic cells in long-term survivors who did not subsequently reject their graft.

Failure of donor marrow engraftment with subsequent autologous recovery has been rarely reported in patients with aplastic anemia.[10-15,29] In most of these patients, cytogenetic studies were not used to confirm the coexistence of donor and host hematopoietic cells prior to rejection. Two of our mixed chimeric patients showed complete autologous recovery, and it may have been the intrinsic ability to recover from severe aplastic anemia that first led to mixed chimerism and then to a gradual displacement of the graft.

A second major finding of the present study is that, censoring for graft rejection, patients with mixed chimerism who maintained their first grafts had a significantly reduced incidence of grade II through IV acute GVHD.

The precise mechanisms leading to mixed chimerism and to the avoidance of acute GVHD in our patients are unknown. A likely explanation is that elimination of host cells requires both the cytotoxic effects of the preparative regimen (in this case cyclophosphamide) and the immunologic reaction of allogeneic donor T cells against residual host immune cells. Donor cells that are more closely matched for non-HLA antigen with the patient may not be able to eradicate host cells, and therefore the persistence of mixed chimerism may be evidence of closer tissue matching between host-donor pairs. Less GVHD in this setting would therefore be expected. Similarly, donor cells associated with less vigorous immune response genes may be less able to eradicate residual host cells, and less GVHD might also be expected in this setting.[30,31] In either case, the inability of donor cells to eradicate host cells would likely allow a rebound in host immunity and therefore an increased risk of graft rejection. Alternatively, persistent host cells might in some way actively suppress donor reactivity against the host. The precise way in which this might occur is unclear but could involve either active suppression or elimination of donor clones by persisting or reemerging host cells.[32] In this clinical setting, the issue of whether putative minor antigens responsible for GVHD might be the same as or different from those responsible for rejection is unresolved.

The observations and conclusion in this study are limited to sex-mismatched donor-recipient combinations prepared for transplantation without TBI since identification of donor-host origin is simplest and most accurate in this setting. We have also limited our analysis to patients prepared for transplant with cyclophosphamide. In the few transplants we have performed for aplastic anemia with TBI, mixed chimerism was seen only once, presumably due to the more intensive immunosuppressive preparative regimen.

We have previously observed that recurrent leukemia is seen less often posttransplant in patients with significant GVHD than in recipients of allogeneic marrow who do not develop GVHD.[33] This observation has been interpreted to mean that one of the targets of the GVH reaction is host leukemic cells. An alternative explanation for the graft-*v*-leukemia effect compatible with the data presented here is that persisting leukemic cells, alone or with other host elements, are themselves capable of suppressing the GVH reaction. Ultimately it will be essential to understand these phenomena more completely since they may provide methods for preventing GVHD and insights into how donor-host reactivity may or may not be manipulated to aid in the treatment of aplastic anemia and leukemia.

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Treatment of Hairy Cell Leukemia With Recombinant Alpha Interferon: I. Quantitative Study of Bone Marrow Changes During the First Months of Treatment

By Georges Flandrin, François Sigaux, Sylvie Castaigne, Christian Billard, Michel Aguet, Michel Boiron, Ernesto Falcoff, and Laurent Degos

Seventeen patients with hairy cell leukemia (HCL) were treated with low doses of recombinant alpha interferon (IFN) for over 4 months. Marked improvement was observed in peripheral blood and bone marrow in 15 of 17 patients. Comparison of pretreatment values and hemograms obtained after 4 months of treatment showed a marked decrease in circulating hairy cells ($P < .01$), a decrease in the number of lymphocytes ($P < .01$), a rise in the number of platelets ($P < .05$), granulocytes ($P < .05$), and monocytes ($P < .01$), and a rise in the hemoglobin level ($P < .01$). Transient reduction in the number of granulocytes was noted during the first month. Correction of thrombocytopenia often appeared within 2 months and usually preceded improvement of anemia, monocytopenia, and neutropenia. Bone marrow biopsy specimens were taken before treatment and 2, 4, and 7 months after its initiation. The volumes occupied by hairy cells, cells of the myeloid lines, and adipocytes were studied by stereological analysis of semithin sections. Decrease in the volume occupied by hairy cells was seen after 4 months of treatment ($P < .01$), and the volume continued to decrease at the seventh month ($P < .05$). Hairy cells were no longer detected on bone marrow biopsies of 4 of 17 patients by the fourth month and in 3 of 8 additional patients by the seventh month. A rise in the volume occupied by normal myeloid cells was visible by the second month of treatment ($P < .01$). Nevertheless, the volume occupied by granulocytes remained lower than in the normal controls ($P < .01$). After an initial increase during the first 2 months of treatment ($P < .01$), the overall cellularity remained unchanged at 4 months and decreased significantly ($P <$ .05) at 7 months. Except for biopsies at 2 months, mean cellularity was below that of control biopsies ($P < .01$).

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QUESADA ET AL recently demonstrated the efficacy of treating seven cases of hairy cell leukemia (HCL) with partially pure alpha (leukocyte) interferon.[1] They obtained three complete and four partial remissions. In order to test the efficacy of treatment with comparable low doses of recombinant alpha interferon (IFN), we started a clinical trial in May 1984 in which 37 HCL patients were progressively entered. This treatment was evaluated by its effects on both peripheral blood and bone marrow However, fibrosis rendered bone marrow study difficult, and satisfactory investigation could be achieved only by using bone marrow biopsies. There were additional problems of correctly identifying the hairy cells and selecting the method of cell evaluation least subject to sampling error. In order to facilitate hairy cell identification and obtain objective evidence regarding alterations of the bone marrow, semithin sections prepared from biopsy specimens embedded in resin were subjected to stereological analysis.[2] This report is based on 17 patients of our series who received treatment for over 4 months and for whom we monitored the changes in peripheral blood and the volumes occupied by hairy cells, myeloid cells, and adipocytes in bone marrow biopsies both before and 2, 4, and 7 months after initiation of treatment.

MATERIALS AND METHODS

Treatment protocol. In May 1984, a nonrandomized study using subcutaneous injections of recombinant alpha interferon from two different suppliers (alpha$_A$ IFN from Hoffman-La Roche, (Basel, Switzerland), 3 × 10^6 U/d, and alpha$_2$ IFN from Schering Corp., 2 × 10^6 U/m^2 in three weekly injections) was started In September 1984, we also began treating patients with partially purified (leukocyte) alpha IFN 3 × 10^6 U/d. Leukocyte alpha IFN was kindly provided by Dr Cantell (Finnish Red Cross). All patients were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent. The initial phase of treatment lasted 7 months. Between May 1984 and March 1985, 37 patients were progressively enrolled in the trial. Thirty one patients were treated with recombinant alpha IFN and six with natural alpha IFN

Patients This report is based on 17 patients for whom treatment with recombinant IFN has exceeded 4 months. Eleven patients had undergone splenectomy at least 6 months previously. Two of the six nonsplenectomized patients did not exhibit splenomegaly prior to treatment; the remaining four patients had splenomegaly of less than 6 cm under the costal margin. Only one patient had prior chemotherapy. Hairy cells were found in the bone marrow of all patients and in the blood of all but one patient. Five patients had hyperleukocytosis (leukocytes > 10 × 10^9/L). Fourteen patients were cytopenic, with decreased levels of hemoglobin (< 12.5 g/dL), granulocytes (< 1 5 × 10^9L), or platelets (< 150 × 10^9/L). Nine patients received alpha$_A$ IFN and eight alpha$_2$ IFN. No significant difference (U test) was found between the two groups as to levels of hairy cells, granulocytes, platelets, or hemoglobin Treatment was discontinued after 4 months for one patient due to total lack of response and for another patient (a woman with a mild form of HCL) because of serious alopecia.

Methods Hemograms and leukocyte counts were done weekly during the first month and at least monthly thereafter. Bone marrow biopsies (BM) were done with a Jamshidi needle in the days immediately preceding treatment and 2, 4, and 7 months after initiation After embedding the specimens in resin (epoxy or glycolmethacrylate), semithin sections (2 μm) were prepared and stained with toluidine blue (Fig 1) or Giemsa. The reticular network was stained by a technique derived from Gomori's method. Stereological analysis (point-counting method) of bone marrow was carried out according to the usual methods,[2] using a Weibel M42 multipurpose grid (objective 100 ×, eyepiece 12.5). For each specimen approxi-

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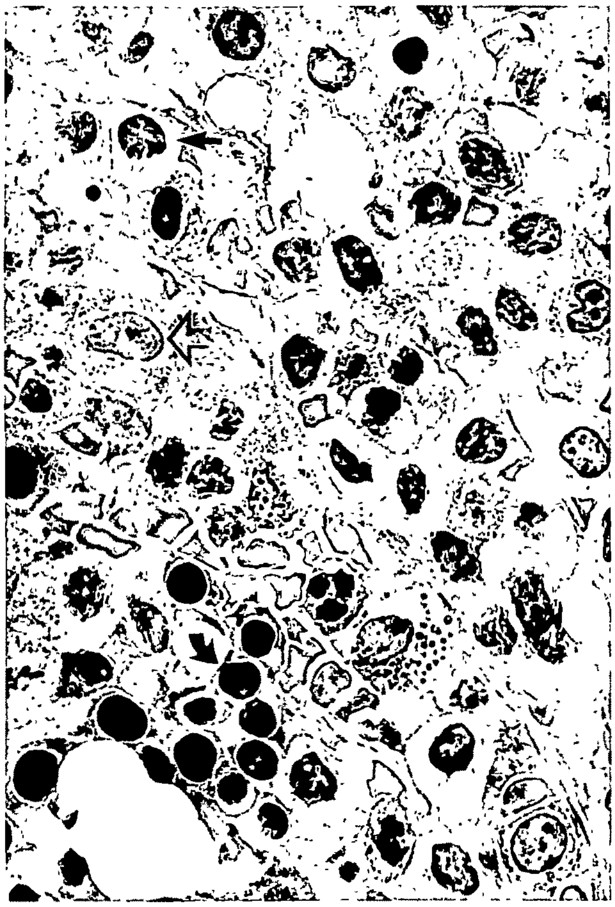

Fig 1. Semithin section of bone marrow biopsy obtained after 4 months of treatment. ←, hairy cells; —→, erythroblasts; ⇐, granulocytes.

mately 1,000 points were counted (1,010 ± 44), giving a probable error of .2%, .3%, .5%, and .7% for the relative volumes (ie, volume density) of 1%, 2%, 5%, and 10%, respectively[2] The analysis was done on randomly selected fields according to a predetermined diagram With counting based on the entire cell (both nucleus and cytoplasm), each cell was classified as one of the following: hairy cell, erythroblast, megakaryocyte, granulocyte, or adipocyte. Lymphocytes, plasmocytes, eosinophils, vascular cells, and unidentifiable cells were grouped together in the category "other cells." Extracellular spaces and other elements of the stroma were also computed As extracellular spaces and fibrosis were obvious in most biopsies, cellularity cannot be defined by reference to adipocytes. We defined cellularity as the sum of hairy cell, myeloid cell, and "other cell" relative volumes Ten normal-appearing marrows were measured as controls (Hodgkins and non-Hodgkins lymphomas without bone marrow infiltration in adults aged 40 to 50 years).

Statistical analysis. Comparisons of the means were tested by Wilcoxon's paired test for paired values and by Mann-Withney's test for unpaired data. Correlations were analyzed by the Spearman correlation test. The P level was set at 5% Data were pooled from patients treated with alpha$_A$ IFN and from those treated with alpha$_2$ IFN For simplicity, both interferons are referred to as IFN in the text.

RESULTS

After 4 months of treatment, we found a decrease in the number of circulating hairy cells ($P < .01$) and lymphocytes ($P < .01$) and a rise in the level of granulocytes ($P < .05$), monocytes ($P < .01$), platelets ($P < .05$), and hemoglobin ($P < .01$). Normalization of anemia (Hb < 12.5 g/dL), granulocytopenia (granulocytes < 1.5×10^9/L), and thrombopenia (platelets 150×10^9/L) was observed, respectively, in 8 of 11, 6 of 12, and 7 of 10 of the initially affected patients. Figure 2 shows the evolution of the mean hematologic values over the first 4 months. During the first month there was a decrease in the number of circulating hairy cells, particularly marked (with the exception of one nonresponder patient) for the hyperleukocytic patients. There was also a transient reduction in granulocytes for all patients during the first month. As shown in Fig 2, in initially cytopenic patients, mean platelet levels increased the first and second months of treatment, while increases in granulocytes and hemoglobin levels were not significant before the end of the second month. The evolution of monocyte levels was somewhat variable, with transient monocytosis commonly cumulating during the third month. In patients who were not initially cytopenic, no significant changes in neutrophil, platelet, or hemoglobin levels occurred after the second month (Fig 2). In the 13 patients who were treated for over 4 months, and results remained good during continued treatment (5 to 7 months) or even improved (five cases).

Hairy cells were no longer detectable in 4 of 17 patients (all treated with alpha$_2$ IFN) after 4 months and in 3 of 8 additional patients (1 treated with alpha$_A$ IFN) after 7 months. All biopsies showed reticular fibrosis. After 4 months of treatment, we observed no clear fibrosis diminution as assessed by conventional morphology. Fibrosis was

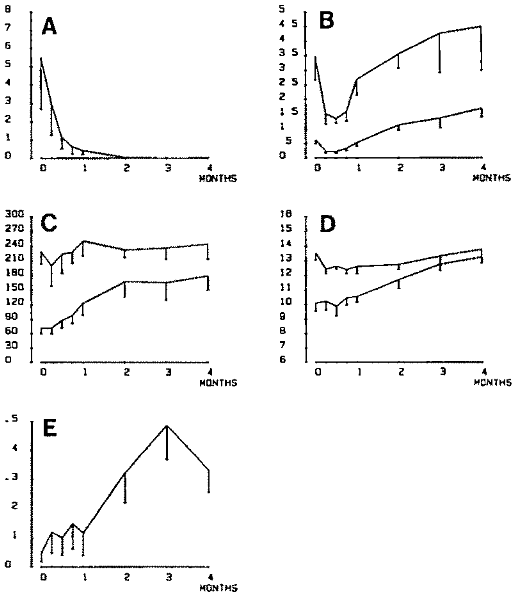

Fig 2. Evolution of hemograms throughout the first 4 months of treatment for the 16 patients with objective responses. (A) HC, hairy cells; (B) PMN, granulocytes; (C) PLT, platelets; (D) HB, hemoglobin; (E) MONO, monocytes. For PMN, PLT, and HB, the lower curve of the graph plots the values for the cytopenic patients and the upper curve those for the noncytopenic patients; cutoffs adopted were PMN, 1.5×10^9/L; PLT, 150×10^9/L, HB, 12.5 g/dL. The plotted points are the means of the parameters; ⊢ is the standard error of the mean (SEM).

always diffuse. In the biopsies showing hairy cell focal invasion, fibrosis seemed greatest in the regions infiltrated by these neoplastic cells. Compared to the initial biopsies, after 4 months all patients showed a significant decrease in the volume occupied by hairy cells ($P < .01$) and an increase in the volumes occupied by cells of the granulocyte ($P < .01$), erythroblast ($P < .01$), and megakaryocyte ($P < .01$) lines No significant changes of volume were found for adipocytes, but a significant increase in overall cellularity ($P < .05$) was observed.

The evolution of the mean stereological parameters is illustrated in Fig 3 A significant decrease ($P < .01$) in the volume occupied by hairy cells was found in month 4 biopsies and became more marked by month 7 ($P < .05$). An increase in the volumes occupied by myeloid cells became distinct by the second month ($P < .01$), but month 4 biopsies showed no further significant change. Month 7 biopsies showed a decrease in the volumes of erythroblasts ($P < .05$), while granulocytic and megakaryocytic cell lines showed no significant changes. The mean volumes occupied by adipocytes remained comparable on initial biopsies and after 2 and 4 months' treatment. By the seventh month, however, an increase was observed in adipocyte volume compared to the month 4 volume for the same patients ($P < .01$). Mean granulocyte volumes, either initially or after 2, 4, and 7 months of treatment (respectively, 2.94 ± 4.79%, 11.12 ± 8.47%, 13.66 ± 8.10%, and 12.86 ± 8.8%) remained significantly ($P <$ 01, U test) below those of controls (26.11 ± 3.68%). This hypoplasia of the granulocytic line was not correlated with the persistence or disappearance of hairy cells and was present in 4 of 7 patients whose biopsies no longer showed any hairy cells The cellularity increased after 2 months ($P <$ 01), remained unchanged after 4 months, and decreased after 7 months ($P < .05$). No statistically significant correlation (Spearman test) was found between platelet, neutrophil blood cell count, hemoglobin level, and BM volumes of megakaryocytic, granulocytic, and erythroblastic series, respectively

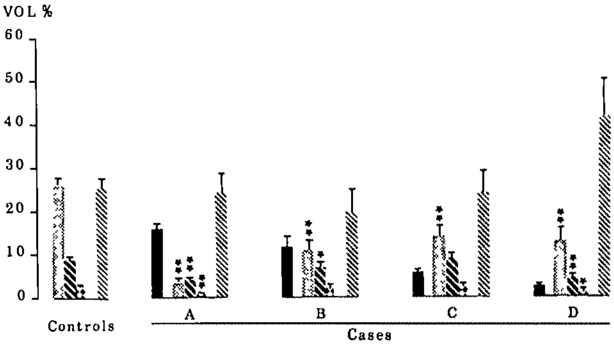

Fig 3. Mean relative volumes occupied by the different constituents of the bone marrow tissue, determined by stereological analysis of semithin sections. Controls: figures for ten bone marrows of normal appearance (see Materials and Methods). Patients: mean volumes (A) before treatment (17 patients), (B) after 2 months of treatment (12 patients), (C) after 4 months of treatment (17 patients), (D) after 7 months of treatment (8 patients). ⊢ is the SEM. ■, hairy cells; □, granulocytes; ▧, erythroblasts; ⊟, megakaryocytes; ▧, adipocytes. The mean volumes for HCL patients were compared with those of the controls (Mann-Withney U test): $^{*}P < .05$, $^{**}P < .01$. Cellularity (as defined in MM) was 22.8 ± 7.6% before treatment, increased ($P < .01$) to 29.7 ± 11.3% after 2 months, remained at 30.0 ± 10.4% after 4 months, and decreased ($P < .05$) to 20.6 ± 11.4% after 7 months of therapy. Cellularity after 4 and 7 months of treatment remained significantly ($P < .01$) below that of the controls (37.8 ± 4.9%).

As a whole, a clear improvement was observed for all but two patients. No improvement was noted for one patient, who was characterized by the presence of hyperleukocytosis, the absence of any myeloid cytopenia with a persistent monocytosis appearing after splenectomy. The response was modest for another patient who had undergone unsuccessful chemotherapy (vinblastine, bleomycin, prednisolone), which was discontinued 1 month prior to treatment with alpha IFN. With the 15 other patients, regardless of the form of alpha IFN administered, distinct improvement of blood, bone marrow, or both was obtained. The splenomegaly present in four patients disappeared during the first 2 months of treatment. It must be stressed, however, that complete normalization of blood and bone marrow parameters (including restoration of myeloid volumes at normal level) were obtained in only one patient. Apparently, splenectomized and nonsplenectomized patients show identical outcomes during the first months of therapy.

DISCUSSION

The management of HCL is still open to controversy. Splenectomy produces improvement of myeloid cytopenias in 30% to 60% of cases and significantly increases survival for patients with severe splenomegaly.[3-5] What remains difficult is the treatment of patients without splenomegaly (28% of 211 cases in our previous series[5]) and of nonresponders to splenectomy (50% in our series). Single-drug therapies, particularly with chlorambucil, often improve anemia and thrombocytopenia but seem to have no effect on neutropenia;[6] the patients remain exposed to infection, which is the most common cause of death and an important prognostic factor[7] Leukapheresis[8] produces only transient improvement. Combination drug therapy is used for the more serious cases (approximately 5% of all cases) and can induce some complete remissions, but at the cost of very high mortality.[10] Quesada's recent demonstration of the efficacy of natural alpha IFN[1] prompted us to start a clinical trial with recombinant alpha IFN for patients with HCL of varying degrees of severity. The unequivocal effects documented in the blood and bone marrow of 15 of 17 patients who were monitored for more than 4 months attest to the rapid action of alpha IFN treatment and are in agreement with results of other concurrent studies.[11-16] Compared to other forms of treatment (other than combination drug therapy), IFN is the only one that achieves the disappearance of hairy cells in the bone marrow in a certain percentage of cases (4 of 17 cases after 4 months and 3 of 8 additional cases after 7 months) and the correction of myeloid cytopenia.

The difficulty of correctly assessing the state of bone marrow during treatment must be stressed. Reticular fibrosis, which interferes with aspiration, persists for several months even after complete remission.[9] Hairy cell infiltration can be studied only by using bone marrow biopsies, and even then, cell identification remains difficult and presents diagnostic problems when the infiltration is moderate. The technique of semithin sections prepared from specimens embedded in resin makes the identification of abnormal cells easier (Fig 1) and permits reproducible stereological studies of the volumes occupied by the different marrow cells. This objective quantitative analysis allows us to establish that the increase in the myeloid cell volumes is rapid, detectable by the end of the second month of treatment. The decline of the hairy cell infiltration generally occurs later, becomes significant only by month 4, and continues to decline until month 7. These results are in contrast with those of the peripheral blood, in which the decrease of hairy cells occurred early, often within the first month, whereas correction of cytopenia (except thrombocytopenia) takes place later, becoming significant only after 3 or 4 months of treatment. The dissociation of blood and marrow results can be observed both at month 4 and at month 7 and compels us to evaluate the treatment by its effects on blood and bone marrow alike.

Quantitative analysis of the biopsies also allows us to show that the volumes occupied by the granulocytic series remained much smaller than in the controls (except for two of 17 cases at month 4 and one additional case of eight at month 7). In addition, 7-month biopsies showed a decrease in cellularity and erythroblast volumes and a clear increase in adipocyte volume. The significance of these findings remains unclear. Both residual disease and interferon therapy may be involved. However, a biopsy represents but a very small fraction of the total marrow. Marrow scans will be performed to search for a peripheral expansion of active hematopoietic marrow. This fact can explain the lack of correlation between blood counts and volumes occupied by the myeloid precursor cells in the bone marrow.

The quality of the clinical results in the long term, the advantage of obtaining complete remission rather than mere normalization of peripheral cytopenias, and the optimum duration of treatment still need to be clarified. Nevertheless, it is already possible to assert that the results obtained to date for the more severe forms (eg, severe pancytopenia with serious recurrent infections: three cases in this study) are of considerable interest since the prognosis without treatment for such patients is poor.[10] Furthermore, the very low growth rate of hairy cells[17] gives us good reason to hope for lengthy remissions for a high proportion of HCL patients.

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Treatment of Hairy Cell Leukemia With Recombinant Alpha Interferon: II. In Vivo Down-Regulation of Alpha Interferon Receptors on Tumor Cells

By Christian Billard, François Sigaux, Sylvie Castaigne, Françoise Valensi, Georges Flandrin, Laurent Degos, Ernesto Falcoff, and Michel Aguet

Interferons (IFNs) initiate their effects by interacting with specific high-affinity cell surface receptors, but little is known about the physiology of IFN receptor interaction in vivo. Treatment of patients suffering from hairy cell leukemia (HCL) with human recombinant alpha IFN results in significant tumor regression, with clinical improvement in a high percentage of cases. To investigate a possible relevance of binding parameters as response markers, IFN receptor interaction on tumor cells responsive to IFN in vivo was studied. Binding of human alpha 2 IFN to circulating hairy cells was analyzed before and during IFN therapy in ten patients selected on the basis of high numbers of peripheral hairy cells. Binding experiments were carried out on Ficoll-Paque fractionated peripheral cell samples containing a majority of hairy cells. All patients reacted to recombinant alpha IFN treatment with a striking decrease in binding capacity within 12 hours after the first injection. As demonstrated by using a monoclonal antibody able to recognize alpha 2 IFN bound to its receptor, this decreased binding capacity was not due to blocking by circulating IFN but rather to a decrease in receptor number. This receptor "down-regulation" was partially reversible after the first IFN injection. However, upon prolonged IFN therapy, all patients displayed a stable state of decreased receptor expression. Down-regulation of IFN receptors can be regarded as a response marker to IFN treatment. This response marker, however, was not correlated with the clinical response within the first months of IFN therapy.

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INTERFERONS (IFNs) are proteins with the common property of protecting cells from viral infection. In addition they exert a variety of pleiotropic effects, such as modulation of various immune functions and inhibition of cell growth.[1-4] Like polypeptide hormones or growth factors, IFN action is initiated by binding to specific high-affinity cell surface receptors.[5,6] IFNs comprise three types (alpha, beta, and gamma), and the alpha type is further subdivided into at least 12 subtypes.[7] While pathways of gamma IFN action seem independent from those of alpha and beta IFN,[8,9] the latter initiate their effects by interaction with presumed common receptors.[9-13] Despite a considerable amount of data on the initial receptor interaction of IFNs and the subsequent steps, including internalization and degradation, major questions on the signal initiated by receptor binding are unresolved. Likewise, the necessity of ligand and/or receptor internalization for interferon action has not been demonstrated unequivocally, and the role of decrease of receptor number induced by IFN binding (called receptor "down-regulation") remains unclear.[5,6,9,14,15] The antitumor potential of IFNs can be mediated by a direct cytostatic action on tumor cells as well as indirectly through stimulation of immune response; in both cases, the mechanisms involved are elucidated only in part.[2] Beyond comparative binding data on various lymphoid cells,[16] little is known as yet on the initial steps of IFN action in vivo. Recently, alpha IFN was reported to induce remission in hairy cell leukemia (HCL).[17] To investigate the role of cell surface receptors as markers for responsiveness of HCL to IFN therapy, IFN binding was studied on tumor cells from HCL patients treated with recombinant alpha IFN. Five of the 17 patients studied in part I of this report and five additional patients who entered the same clinical trial were selected on the basis of high numbers of circulating hairy cells before onset of IFN therapy. Number of receptor sites for alpha IFN and the affinity constant were determined before and during IFN therapy by studying the binding of ^{125}I-labeled human alpha 2 IFN.[10] In addition, the degree of receptor occupation under IFN treatment was studied with a monoclonal antibody that recognizes alpha 2 IFN bound to its receptor.

MATERIALS AND METHODS

Patients and treatment protocol. Experiments were carried out with ten patients with hairy cell leukemia who entered the trial described in part I of this report. The patients were selected on the basis of high number of peripheral hairy cells before onset of therapy (>10 × 10^9 leukocytes/L) Based on peripheral blood and bone marrow data, clinical progress of patients 2, 4, 8, 9, and 21, for whom treatment has exceeded 4 months, is described in part I of this study. All patients were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent.

Patients 2, 4, 25, and 29 were treated with daily subcutaneous injections of 3 × 10^6 units of recombinant human alpha 2 IFN (Hoffman-La Roche, Basel, Switzerland). Patients 4, 8, 21, 27, 36, and 38 were treated with 2 × 10^6 U/m^2 of recombinant human alpha 2 IFN (Schering Corp, Kenilworth, NJ) in three weekly subcutaneous injections. These two IFNs differ in only one amino acid[7] and are referred to as recombinant alpha IFN in the following text

Binding assay. Peripheral blood cells, including a majority of hairy cells, were enriched by fractionation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol. The percentages of hairy cells obtained under these conditions for each binding experiment are shown in Table 1. Subsequently, cells were washed three times at 4 °C with Hank's balanced salt solution (HBSS) containing 10 mmol/L HEPES with 10% fetal calf serum (KC Biological, Lenexa, Kan) and resuspended in this buffer at a density of 2 × 10^7 cells/mL. To determine saturation curves, binding experiments were carried out in U-bottomed microtiter plates at a cell density of 10^7 cells/mL (200 μL/well) as described elsewhere[18] All incubations with ^{125}I-labeled human alpha 2 IFN were for 90

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Table 1. Percentage of Hairy Cells in Peripheral Blood Mononuclear Leukocytes Isolated for Binding Assays During IFN Therapy

Patient No	Weeks of Treatment	Hours After Each IFN Injection				Patient No	Weeks of Treatment	Hours After Each IFN Injection			
		0	6	12	24			0	6	12	24
9	0	90	93	97	97	8	0	99	88	99	100
	1	68	87	—	—		1	94	68	—	—
	2	85	76	—	—		2	55	42	—	—
	3	62	ND	—	—						
	4	33	—	—	—	38	0	88	92	83	93
29	0	ND	91	97	ND	27	0	98	97	95	98
	1	99	95	—	—		1	77	96	—	—
	2	99	95	—	—		2	ND	92	—	—
	3	95	93	—	—		3	92	ND	—	—
							4	99	91		
25	0	75	97	91	79						
	1	81	85	—	—	36	0	ND	84	ND	90
							1	ND	56	—	—
2	0	64	81	ND	ND		2	5	ND	—	—
	2	81	ND	—	—						
	3	63	57	—	—	4	0	94	97	98	100
							1	91	90	—	—
21	0	97	98	88	93		2	84	96	—	—
							3	79	ND	—	—
							4	77	ND	—	—

HCL patients were treated according to the protocols described in Materials and Methods For each binding experiment, peripheral blood mononuclear leukocytes were fractionated on Ficoll-Paque gradient. Other cells present in these preparations were lymphocytes and occasionally monocytes. ND, not determined

minutes at 4 °C. Specific binding of ^{125}I–alpha 2 IFN was measured at six different IFN concentrations: 10,000, 5,000, 2,500, 1,250, 625, and 300 U/mL. Nonspecific binding was determined by simultaneous addition of 2×10^5 U/mL of unlabeled human alpha 2 IFN. Binding constant (Kd) and number of binding sites were extrapolated by Scatchard analysis of saturation curves as described previously [18]

Since blood samples had to wait for variable periods of time (up to 12 hours) at 4 °C prior to IFN binding experiments, it was important to ascertain the variability of binding data during these intervals. On cells kept at 0 °C to 4 °C in balanced salt solution, receptor expression and binding affinity remained virtually unchanged for periods up to 48 hours. Also, cell fractionation on Ficoll-Paque did not modify the binding data (data not shown)

Mouse monoclonal antibody against human recombinant alpha 2 *IFN* Mouse monoclonal antibodies against human recombinant DNA alpha 2 IFN were raised as described elsewhere.[19] These antibodies were characterized with regard to IFN subtype specificity, neutralization of antiviral activity, and the capacity to recognize human alpha 2 IFN specifically bound to its cell surface receptors.[20] For the indirect binding assay described below, an antibody (I/11) specific for alpha 2 IFN in a solid-phase radioimmunoassay was selected.[19] Although this antibody did not neutralize the biological activity of alpha 2 IFN it was able to recognize alpha 2 IFN specifically bound to its cell surface receptors on human lymphoid Daudi cells pretreated with alpha 2 IFN. Nonspecific binding was determined by simultaneous addition of excess human beta IFN, which binds to the same receptors as alpha IFN[9,12,13] but is not recognized by the monoclonal antibody I/11. Binding of monoclonal antibody I/11 was revealed by addition of an ^{125}I-labeled rabbit antimouse IgG antibody as described below.

Indirect binding assay. Peripheral blood cells were prepared as described above and adjusted to 2×10^7 cells/mL in the same buffer. To detect receptor-bound alpha 2 IFN, the cells were incubated for 90 minutes at 4 °C with the mouse monoclonal antibody (I/11) capable of recognizing alpha 2 IFN specifically bound to its cell surface receptors, which has been described above and elsewhere.[19] Control experiments were carried out with a nonspecific mouse monoclonal antibody (I/0). Cells were then washed twice, and bound mouse antibody was revealed by incubation for 90 minutes at 4 °C with an ^{125}I-labeled rabbit antimouse IgG antibody in the presence of 1% normal rabbit IgG to block Fc receptors. This rabbit antimouse IgG antibody was purified by affinity chromatography on mouse IgG coupled to Affigel-10 (Biorad, Richmond, Calif) and labeled with [^{125}I] sodium iodide (CEA, Gif sur Yvette, France) to a specific radioactivity of 15 to 30 μCi/μg protein. Briefly, 1 mCi was added to 25 μg IgG in 100 μL phosphate-buffered saline (PBS) and the iodination initiated by addition of 10 μL of chloramine T adjusted to 500 μg/mL in PBS. After 45 seconds the reaction was stopped by addition of 10 μL of sodium metabisulfite at 500 μg/mL in PBS Unreacted iodine was eliminated by chromatography of the reaction mixture on a prepacked Sephadex G-25 PD-10 column (Pharmacia) To control the capacity of antibody I/11 to recognize receptor-bound alpha 2 IFN, cells were preincubated for 90 minutes at 4 °C with unlabeled alpha 2 IFN at a saturating concentration of 10,000 U/mL prior to incubation with mouse monoclonal antibodies I/11 and I/O, respectively All incubations were carried out as for the direct binding assay in U-bottomed microtiter plates at a cell density of 10^7 cells/mL in a volume of 200 μL/well. The labeled rabbit antibody was adjusted to approximately 200,000 cpm/well.

IFN assay. Interferon activity was assayed by inhibition of the cytopathic effect of vesicular stomatitis virus (VSV) on bovine MDBK cells (Flow Laboratories,Bethesda, Md) Briefly, confluent monolayer cultures of MDBK cells were incubated for 24 hours at 37 °C with interferon samples of twofold dilution series. Subsequently the cell monolayers were challenged with .1 $TCID_{50}$/100 μL VSV. Inhibition of cytopathic effect was determined after 24 hours' incubation with the challenge virus. Human recombinant DNA alpha 2 IFN served as a reference with a specific activity of 2×10^8 U/mg protein. Titers were standardized to the NIH human leukocyte interferon No. GA 902-530 and are expressed in international units (U/mL).

RESULTS

High-affinity binding of ^{125}I-labeled alpha 2 IFN to specific cell surface receptors of hairy cells was measured immediately before and at various intervals after onset of recombinant alpha IFN therapy. The proportion of hairy cells in peripheral blood is listed in Table 1 for each determination of receptor number. Figure 1 shows the evolution of the number of high-affinity alpha 2 IFN receptors in the first 4 weeks of IFN therapy. Initial numbers of high-affinity alpha 2 IFN receptor sites per cell ranged from 150 to 1,100 in a cell population with more than 90% hairy cells (Table 1). All patients exhibited a 70% to 80% decrease in the number of sites per cell as short as 6 to 12 hours after the first recombinant alpha IFN injection. Twenty-four hours after the first injection, the binding capacity was restored partially in most patients, while it remained at less than 20% of the pretreatment level in two cases (Fig 1). This recovery was only temporary, since binding experiments performed at weekly intervals after onset of therapy revealed a binding capacity consistently below 25% of the pretreatment level. After the second week of treatment, the binding capacity remained virtually unchanged before and six hours after recombinant alpha IFN injections. A decreased binding capacity was also observed in patient 2, who did not respond clinically to IFN therapy. Serum IFN was assayed at each point of determination of binding capacity. While no IFN activity was detectable before onset of therapy, recombinant alpha IFN treatment yielded serum levels between 0 and 40 U/mL. Titers did not correlate significantly with the decreased binding capacity (data not shown).

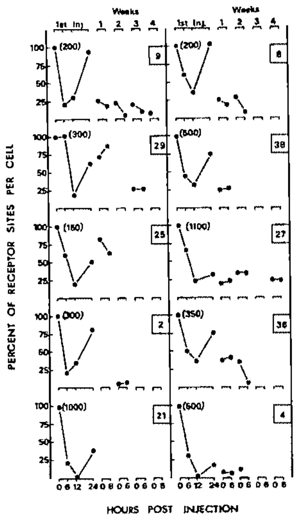

Fig 1. Time course of alpha receptor IFN receptor expression during recombinant alpha 2 IFN treatment of HCL. The results obtained with ten donors are represented individually with time of measurement on the abscissa (weeks after onset of IFN therapy, hours before and after IFN injection) and numbers of specific high-affinity binding sites per cell on the ordinate. These were extrapolated from Scatchard analysis of saturation curves as indicated in Materials and Methods. Saturation curves were based on triplicate values with standard deviations usually less than 10%. The number of receptors per cell before therapy is indicated in parentheses and represents 100% binding capacity. Patient number is indicated in the square at the top right of each graph.

In contrast to the number of binding sites, binding constants (Kd) determined before IFN treatment did not differ significantly and ranged from 1.4 to 6.7 × 10^{-10} mol/L. As exemplified for one case in Fig 2, the affinity of the residual binding sites remained unchanged after IFN injection as compared to the binding properties before treatment.

To distinguish between blocking of receptors by circulating recombinant alpha 2 IFN and decreased expression of high-affinity receptors, the degree of receptor occupation before and six hours after IFN injection was studied in an indirect binding assay with a monoclonal antibody (I/11) directed against alpha 2 IFN bound to its receptor[19] or a control antibody (I/0). Binding of these antibodies was revealed by use of an ^{125}I-labeled rabbit antimouse IgG antibody. As shown in Fig 3, virtually the same amount of radioactivity was bound to hairy cells immediately before and six hours after the first alpha 2 IFN injection with both specific and control antibody. These results suggest that the decreased binding capacity after IFN injection as revealed in the direct binding assay (Fig 1) was not due to occupied receptors. As a control, the IFN binding capacity before and six hours after IFN injection was also determined in the indirect assay. For this purpose the cells were preincubated with a saturating amount of alpha 2 IFN (10,000 U/mL) prior to incubation with the monoclonal antibodies I/11 or I/0, respectively. The results (Fig 3) are consistent with those obtained in direct binding experiments depicted in Fig 1. Thus six hours after the first injection, the IFN binding capacity of hairy cells from patients 4 and 25 was decreased to the same extent in both assays (Fig 3A and B), while the binding capacity of cells from patient 29 remained unchanged before and six hours after the first IFN injection (Fig 3C). As exemplified with patient 2, the IFN binding capacity of peripheral hairy cells remained low, and no bound IFN was detectable after 10 weeks of IFN therapy

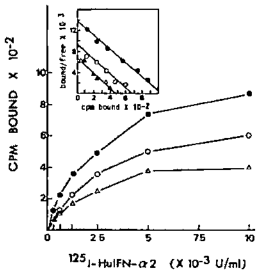

Fig 2. Specific saturable binding of ^{125}I-labeled alpha 2 IFN to hairy cells from patient 38 before (●), 6 hours after (△), and 24 hours after (○) the first IFN injection. The cells were incubated for ninety minutes at 4 °C with various concentrations of ^{125}I-labeled alpha 2 IFN. Nonspecific binding was determined by simultaneous addition to labeled IFN of 2 × 10^5 U/mL unlabelled alpha 2 IFN. Each point represents the mean value of triplicates. Standard deviations were less than 10%. The insert represents the Scatchard plots of the specific saturation curves: The apparent affinity constant as extrapolated from the slope of the curves was approximately 2 × 10^{-10} mol/L. The number of receptor sites per cell was calculated from the extrapolated total number of bound counts per minute on the abscissa.

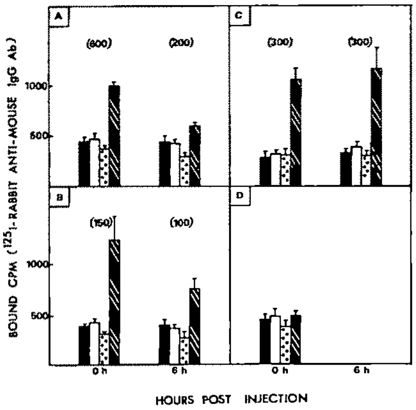

Fig 3. **Indirect detection of alpha 2 IFN bound to cell surface receptors on hairy cells from alpha 2 IFN–treated patients. Peripheral hairy cells were isolated immediately before and six hours after the first alpha 2 IFN injection from patients 4 (A), 25 (B), and 29 (C) and after the tenth week of therapy for patient 2 (D). Specifically, bound alpha 2 IFN was detected by addition of mouse monoclonal antibody I/11 capable of recognizing receptor-bound alpha 2 IFN, as compared to binding of a nonspecific antibody I/0. Binding of these antibodies was revealed by addition of a ^{125}I-labeled rabbit antimouse IgG antibody. To control IFN binding capacity, cells were preincubated with a saturating amount of alpha 2 IFN prior to incubation with monoclonal antibodies I/11 or I/0, respectively. The number of high-affinity alpha 2 IFN receptor sites per cell determined from direct binding-assay is indicated in parentheses and was not detectable in the case of patient 2 (D). ■, cells incubated with control antibody I/0; □, cells preincubated with 10,000 U/mL alpha 2 IFN before incubation with antibody I/0; □, cells incubated with antibody I/11 capable of recognizing alpha 2 IFN bound to its receptor; ▧, cells preincubated with 10,000 U/mL alpha 2 IFN before incubation with antibody I/11.**

(Fig 3D). Consistent results were obtained in the other cases (data not shown). These results suggest that the decreased binding capacity observed in all patients was due to decreased receptor expression rather than to receptor occupation by circulating recombinant alpha IFN.

DISCUSSION

The efficacy of IFN therapy for tumors has been widely investigated over the past few years.[21,22] Due to the poor understanding of mechanisms involved in IFN-mediated tumor regression,[2] predictive parameters for responsiveness of tumor cells to IFN have not been defined as yet. Though presence of IFN receptors appears indispensable for direct IFN action, tumor regression might be mediated by the host, as suggested by the in vivo suspectibility of mouse L1210R cells,[23] which are resistant to the direct effects of alpha and beta IFN in vitro and lack specific receptors.[24] Resistance to IFN can be associated with altered receptor expression and a lower binding affinity[25]; however, resistance to IFN was also observed despite apparently normal receptor expression and also normal induction of response markers such as (2′-5′-)oligo(A) polymerase.[26,27] Further functional characterization of mechanisms activated upon IFN-receptor interaction and particularly of proteins induced in response to IFN[8] will help to elucidate the various forms of IFN resistance and to define markers required for expression of a sensitive phenotype.

The present study was carried out to investigate the initial steps of direct IFN action of hairy cells, which are responsive to alpha IFN treatment in vivo.[17] High-affinity cell surface receptors with binding properties identical to those described for normal peripheral lymphoid cells[13] were found on circulating hairy cells of all ten patients included in the present study. Although pretreatment numbers of detectable binding sites showed up to sevenfold variations (Fig 1), they were of the same order of magnitude as on normal peripheral lymphocytes.[16] After onset of recombinant alpha IFN treatment (see treatment protocol in Materials and Methods), all patients showed a marked decrease of detectable alpha IFN receptors on circulating tumor cells (Fig 1). To distinguish between specific blocking and a decreased number of receptors, bound alpha 2 IFN was revealed indirectly by use of a monoclonal antibody against alpha 2 IFN, which recognizes an epitope still accessible upon receptor binding. Tumor cells from different patients were investigated using this approach immediately before and six hours after the first recombinant alpha IFN injection, as well as upon prolonged IFN therapy (Fig 3). Clearly, the results indicate that the loss of binding sites as early as six hours after onset of therapy was not due to receptor occupation but rather to a decreased receptor expression, called "down-regulation." Alternatively, a pronounced drop in binding affinity may have also accounted for decreased binding of labeled alpha 2 IFN and could not have been distinguished from decreased receptor expression. As shown in Fig 2, the binding affinity of the residual binding sites was unchanged as compared to the data before IFN therapy. Prolonged IFN treatment of cells in vitro may lead to selection of resistant cells.[28] Since a decreased IFN binding capacity was observed rapidly after onset of IFN treatment, selection of tumor cells lacking IFN receptors seems unlikely.

Binding-induced down-regulation of receptor expression was observed in all patients over the entire period of IFN therapy, resulting in marked tumor regression in 9 of 10 patients. Although in most patients receptor expression recovered to a variable proportion within 24 to 48 hours after the first recombinant alpha IFN injection, a permanent "down-regulated" state was established at the latest after the second week of IFN treatment (Fig 1). Down-regulation of alpha IFN receptors, therefore, seems to reflect a continuous response to IFN at the receptor level, indicating that cells remain responsive to IFN despite a low degree of high-affinity receptor expression. After 3 to 4 weeks of treatment, the number of hairy cells in peripheral blood was markedly decreased in most patients (see part 1). However, decreased expression of high-affinity receptors was observed independently of the percentage of tumor cells in the mononuclear cell-enriched fraction (Fig 1, Table 1), suggesting that receptor down-regulation can occur in the majority of circulating leukocytes. Since in most cases the proportion of hairy cells did not vary significantly within the first 2 weeks of treatment (Table 1), receptor down-regulation can hardly be attributed to shifts in cell populations.

Down-regulation might reflect a shift in the dynamic equilibrium between receptor expression and receptor processing.[29] The fraction of occupied receptors at a given concentration of free ligand depends proportionally on the number of expressed receptors.[18] Therefore, receptor down-regulation could result in a decreased sensitivity of the target cell. However, a maximum biological response might require occupation of only a small fraction of accessible receptors, the majority of expressed receptors being "spare."[5] Down-regulation of insulin receptors seems to correlate with insulin resistance in some forms of type II diabetes,[30,31] but it is not clear whether this mechanism could be more generally responsible for decreasing target cell sensitivity for polypeptide ligands. Recently, human Daudi cells rendered resistant to human alpha IFN by prolonged incubation with IFN[27] were shown to "down-regulate" their receptors in the same way as sensitive Daudi cells.[25]

Preliminary results seem to indicate that expression of gamma IFN receptors in hairy cells remains unaffected by alpha IFN treatment (data not shown), in agreement with previous results.[32] Down-regulation occurs rapidly at interferon doses that yield barely detectable serum levels. Thus down-regulation of alpha/beta IFN receptors in vivo could provide a sensitive early response marker, not only for monitoring alpha IFN administration but also to reveal endogenous IFN production in untreated patients with the possibility of distinguishing between alpha and beta IFNs *v* gamma IFN in situations of low or undetectable serum IFN.

Degree and time course of alpha IFN receptor down-regulation were similar in all patients, independent of clinical response to the treatment. The results presented here seem to indicate, therefore, that neither initial IFN receptor levels nor down-regulation is of predictive importance in IFN therapy of HCL. Still, to evaluate the role of receptor down-regulation and its relevance as a response marker for IFN therapy in vivo, it seems important to reevaluate this parameter after prolonged IFN therapy and to extend similar studies to additional cases of IFN-resistant HCL and also to IFN trials in other leukemic diseases.

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CONCISE REPORT

Pathways for the Reduction of Oxidized Glutathione in the *Plasmodium falciparum*-Infected Erythrocyte: Can Parasite Enzymes Replace Host Red Cell Glucose-6-Phosphate Dehydrogenase?

By Eugene F. Roth, Jr., Seymour Schulman, Jerome Vanderberg, and Julie Olson

Plasmodium falciparum-infected human red cells possess at least two pathways for the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH): (1) the glucose-6-phosphate dehydrogenase (G6PD) pathway and (2) the glutamate dehydrogenase (GD) pathway using glutamate as a substrate. Uninfected erythrocytes lack the GD pathway. The NADPH generated can be used to reduce oxidized glutathione (GSSG), which accumulates in the presence of an oxidative stress. In red cell G6PD deficiency, this pathway is reduced or absent, and the host cells as well as the parasites within them are vulnerable to oxidant stress. In view of the presence of the GD pathway in parasitized red cells and the recent description of a parasite-derived G6PD enzyme, we have asked whether the pathways for the reduction of GSSG provided by the parasite can substitute for the host G6PD in red cells deficient in G6PD activity. We have devised a functional assay in which the reduction rate of GSSG is monitored in the presence of buffered infected or control red cell lysates and substrates. Infected G6PD-deficient erythrocytes were obtained from in vitro cultures after a single prior growth cycle of the parasites in G6PD deficient cells to eliminate contaminating normal red cells. The results show that only parasitized red cells can reduce GSSG via the GD pathway. In parasitized G6PD Mediterranean red cells (completely G6PD-deficient), there is a detectable GSSG reduction via the G6PD pathway, not found in uninfected lysates from the same individual. In G6PD A$^-$ (African type, featuring partial deficiency), a small increment in the G6PD-dependent reduction of GSSG can also be detected. However, when compared to G6PD normal red cells, the activities from the parasite-derived pathways are small and could not be considered substitutes for normal host enzyme activity. It is concluded that while the plasmodium provides additional pathways for the generation of NADPH that may serve its own metabolic needs, the host red cells and hence the parasite itself remain vulnerable to oxidant stress.

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OXIDANT STRESS is often invoked as a mechanism for the inhibition of malaria parasite growth in vivo.[1,2] This mechanism is also used frequently to explain the selective advantage of red cell glucose-6-phosphate dehydrogenase (G6PD) deficiency in malarious parts of the world, particularly wherever *Plasmodium falciparum* is prevalent. In G6PD deficiency, the erythrocyte cannot generate enough NADPH to maintain glutathione in the reduced form when challenged by an oxident stress. Glutathione reductase (GR), the enzyme responsible for the reduction of oxidized glutathione (GSSG), has an absolute requirement for NADPH. The depletion of reduced glutathione (GSH), a protective substance that is also a cofactor in some enzymatic processes, may result in cell injury and parasite death. Previously, it had been demonstrated that malaria-infected red cells contain at least one additional pathway, glutamate dehydrogenase (GD), for the generation of NADPH that could be used to drive the reduction of GSSG,[3] and recently, Usanga and Luzzatto have described an inducible parasite form of G6PD in cultures of *P falciparum* adapted to growth in G6PD-deficient red cells.[4] This enzyme, of course, could also serve to produce NADPH for the reduction of GSSG. The presence of a parasite G6PD enzyme had also been suggested earlier.[5] The two pathways for the reduction of GSSG may be summarized as follows:

(1) G6PD pathway:

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{6-phosphogluconate} + \text{NADPH}$$

$$\text{GSSG} + \text{NADPH} \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

(2) GD pathway:

$$\text{Glutamate} + \text{NADP}^+ \xrightarrow{\text{GD}} \alpha\text{-ketoglutarate} + \text{NH}_3 + \text{NADPH}$$

$$\text{GSSG} + \text{NADPH} \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

where NADP$^+$ is oxidized nicotinamide adenine dinucleotide phosphate.

We have asked whether the alternate parasite-derived pathways for GSSG reduction could substitute for the normal erythrocyte G6PD activity. To answer this question, we have devised a functional assay for the reduction of GSSG using infected red cell lysates, substrates, and cofactors for each of the pathways. Although these methods do not allow calculation of classical kinetic parameters of enzyme function, they permit estimation of the relative quantitative importance of each pathway. In addition, they serve as a

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confirmatory method for the more direct enzyme assays, which are technically difficult when only minute traces of enzymes are present.

MATERIALS AND METHODS

The assay system for the estimation of GSSG reduction rates consisted of 100 mmol/L Tris buffer, pH 7.5 or 7.0 as indicated, with 1 mmol/L EDTA to which was added NADP$^+$, GSSG, and either monopotassium glutamate or glucose-6-phosphate (monosodium salt) (G6P), all at a final concentration of 400 μmol/L. This pH range was chosen because it spans the conditions likely to be found in intact red cells. Since no significant differences were found at these pH extremes, only results for pH 7.5 are reported. The mixture was prewarmed in a stoppered 10-mL Erlenmeyer flask at 37 °C on a shaking water bath, and aliquots (10 to 100 μL) of the freeze-thawed red cell lysate and distilled water were added to bring the final volume to 1.0 mL. Incubation was continued usually for 15 minutes (but up to 120 minutes if needed), and the reaction was stopped by addition of an equal volume of cold meta-phosphoric acid-EDTA-NaCl solution used for the extraction of GSH according to the method of Beutler.[6] The reduction of GSSG was linear for the incubation times used, and the loss of the GSH produced was less than 1%. The supernatants were clarified by centrifugation and then assayed for GSH by the colorimetric method of Beutler,[6] modified to accommodate small volumes. For each hemolysate and for each substrate, two blanks were incubated to account for any nonspecific reduction of GSSG and to detect residual background GSH in the hemolysates. One blank lacked substrate (glutamate or G6P), and another lacked GSSG and NADP$^+$. In general, blanks displayed absorbances at the appropriate wavelength (412 nm), which were not more than 3% of the complete system with enzymes, substrates, and cofactors. When no detectable GSSG-reducing activity was observed, subtraction of the blank values did not result in "negative values" In some experiments, exogenous glutathione reductase was added to ensure that this enzyme would not be the limiting factor in the reaction cascade. Accordingly, Baker's yeast GR type III (Sigma Co, St Louis) was obtained as an ammonium sulfate suspension and dialyzed against the Tris-EDTA buffer to which 10 μmol of flavine adenine dinucleotide (FAD) was added as a stabilizer. In some experiments, .88 Sigma units of GR (Sigma Chemical, St Louis) was added to the incubation flask, but the addition of this enzyme generally did not affect the rate of GSSG reduction (one Sigma unit is defined as the reduction of 1.0 μmol coenzyme A–glutathione mixed disulfide/min at pH 5.5 at 25 °C) Removal of the ammonium sulfate by dialysis was necessary because NH$_4^+$ and/or NH$_3$ was inhibitory for GD.

Plasmodium falciparum (FCR–3 strain) was cultured in normal and G6PD-deficient red cells as described by Jensen and Trager[7] and enriched for later parasitic stages (trophozoites and schizonts) by sedimentation with gelatin.[8] Plasmodium cultures were not adapted to growth in G6PD-deficient cells for a number of cycles before harvesting the cells for the assay. However, to avoid contaminating G6PD-deficient red cells with significant numbers of normal erythrocytes, parasites at high parasitemias (obtained by gelatin sedimentation) were added to G6PD-deficient cells for a single growth cycle. When these parasites had attained the later stage of trophozoite/schizonts, another gelatin sedimentation was performed, and the parasite-rich layer was added to a fresh aliquot of G6PD-deficient cells. The biochemical studies were performed after gelatin sedimentation of this second aliquot of cells (at the end of a total of two growth cycles). Thus admixed normal red cells were not harvested for the biochemical studies. The cells were washed three times in the Tris-EDTA buffer used for the assay and counted on a Model Z Coulter Counter. After the addition of NADP$^+$ (1 mmol/L) and FAD (10μmol/L) to stabilize G6PD and GR, respectively, the packed cells were lysed by two freeze-thaw cycles.

Hemoglobin (Hb) content of the lysates was determined by the cyanmethemoglobin method. Blood was obtained from known G6PD-deficient donors used for previous studies.[9,10] Both G6PD Mediterranean and G6PD A$^-$ types were used in this study, and a spectrophotometric kinetic assay method to measure G6PD activity was used as described previously.[9,10] The red cells with G6PD Mediterranean displayed no spectrophotometrically detectable enzyme activity, whereas the G6PD A$^-$ red cells contained 1.1 U/g Hb of enzyme activity (norman range, 8.98 ± 77 SD). The sensitivity limits of the GD pathway were tested by incubating purified GD from bovine liver (type III, Sigma Corp, St Louis) with appropriate substrates, including the dialyzed yeast GR.

The sensitivity limits for G6PD were tested by diluting a normal red cell lysate of known G6PD activity and incubating it with appropriate substrates as described. In this method, as little as one thousandth of the normal red cell G6PD activity could be detected, and as little as 50 Sigma microunits of bovine liver GD/mL could be measured via reduction of GSSG. (One sigma unit of GD is defined as the reduction of 1.0 mmol α-ketoglutarate to L-glutamate/min at pH 7.3 at 25 °C). Results for the lysates were expressed as μmol GSH generated/g Hb/h. This method of expressing GSSG reduction rates was adopted because preliminary experiments indicated that although there is a change in MCH (mean corpuscular hemoglobin) during parasite growth, the cyanmethemoglobin method is not sensitive to the decline in hemoglobin concentration. This phenomenon (which will be the subject of a separate report) occurs because that portion of the hemoglobin converted to the malarial pigment hemozoin has considerable absorbance at the same wavelength as that used for the measurement of cyanmethemoglobin Since Drabkin's reagent measures the concentration of cyanmethemoglobin and hemozoin together, only very minor changes are seen in the infected red cell. Thus, for the purpose of a constant denominator in enzyme measurements, the cyanmethemoglobin method is valid All spectrophotometric measurements were made on a Gilford model 240 UV-visible recording spectrophotometer

To test the role of young red cells in the reduction of GSSG, red cells from normals and a G6PD-deficient Mediterranean donor were separated by density according to the method of Vettore et al[11] on Percoll-Renografin gradients. Three layers were defined—top, middle, and bottom—and these were lysed and studied for GSSG-reducing activity in the presence of glucose-6-phosphate and NADP$^+$.

RESULTS

Lysates from infected erythrocytes of all genotypes possessed a measurable capability to reduce GSSG in the presence of glutamate by means of the enzyme glutamate dehydrogenase. No glutamate-dependent activity was found in the uninfected control cells (Table 1). This finding demonstrates that the parasites were disrupted by the freeze-thaw cycles and that release of intracellular material had occurred. The presence of this enzyme in a number of plasmodia including *falciparum* has been described previously.[3,12]

As expected from spectrophotometric data on red cell G6PD enzyme activity, considerable glucose-6-phosphate–dependent reduction of GSSG occurred in the presence of normal red cell lysates, and no GSSG reduction occurred in the presence of lysates from G6PD Mediterranean cells (Table 1). Lysates from G6PD A$^-$ were capable of reducing

Table 1. The Reduction of Oxidized Glutathione by Lysates of *P falciparum*–infected Red Cells Using Glutamate or Glucose-6-phosphate as Substrates

Lysate	Parasitemia (%)	Glutamate (µmol GSH/g Hb/h)	Glucose-6-PO$_4$ (µmol GSH/h/gHb)
Normal infected (3)	38%	25.4 ± 1.3 SD	727.0 ± 35.2 SD
Normal control (2)	—	0.0	785.0
G6PD Mediterranean infected	30%	21.0	28.9
	60%	73.5	82.7
G6PD Mediterranean control (3)	—	0.0	0.0
G6PD A$^-$ infected	38%	38.6	221.0
	72%	71.5	162.0
	51%	48.0	209.0
G6PD A$^-$ control (3)	—	0.0	159.0 ± 11.9 SD

Numbers in parentheses indicate numbers of different blood donors

GSSG in the presence of glucose-6-phosphate at a reduced rate of about 15% to 20% of the normal rate, which is comparable to their relative enzyme activity as measured by direct kinetic assays. Of great interest is the presence of detectable G6PD activity (as detected by reduction of GSSG) in lysates from *infected* G6PD Mediterranean red cells. Since the uninfected cell lysates from the same donor contained no detectable activity, it is concluded that the enzyme responsible for this activity is derived from the parasites.

Red cell age (density) is an important determinant of G6PD-dependent GSSG-reducing activity in normal erythrocytes (Table 2). The reduction rates of the most buoyant cell layer exceeded the rates of the bottom (most dense) layer by 100 to 200 µmol GSH/g Hb/h. However, when top and bottom layers of G6PD-deficient cells were compared in the same assay, a difference of only 8 µmol GSH/g Hb/h was observed (Table 2). This finding suggests that the G6PD-dependent GSSG reduction rates seen in infected G6PD-deficient cells (Table 1) were not related to preferential invasion of young red cells by plasmodia.

These observations are consistent with the data of Usanga and Luzzatto,[4] who detected G6PD activity in *P falciparum* grown in the same kind of G6PD-deficient red cells. There is also a small increment in glucose-6-phosphate–dependent GSSG reduction seen in parasitized G6PD A$^-$ cells, which is consistent with the coexistence of parasite- and host-derived G6PD enzymes (Table 1). It should also be noted that the increments in GSSG reductive capacity attributed to parasite enzymes does not approach levels for G6PD-dependent activity in normal, uninfected cells. For example, if the activity for the reduction of GSSG in the presence of glucose-6-phosphate in the infected G6PD Mediterranean sample with 60% parasitemia is extrapolated to 100% parasitemia, the value amounts to 138 µmol GSH produced/g Hb/h. This value is still in the deficient range, although it now resembles the African type of G6PD deficiency more than the Mediterranean type. Similar calculations for GD activity yield similar small increments in GSSG reductive capacity. Thus, while the parasitized G6PD-deficient red cells clearly have some GSSG reductive capacity, it does not attain levels found in normal erythrocytes.

Table 2. The Effect of Red Cell Age on G6PD-dependent Reduction of Oxidized Glutathione in Uninfected Normal and G6PD-deficient Red Cells

	Reduction of GSSG (µmol GSH/gHb/h, mean ± SD)	
Density Gradient Layer	Normal RBCs	G6PD-deficient RBCs
Top (n = 4)	1134 ± 116	9.7 ± 5.5
Middle (n = 4)	978 ± 129	1.7 ± .6
Bottom (n = 4)	927 ± 92	1.7 ± .5
Whole blood (n = 4)	1086 ± 44	2.0 ± .6

When the same experiments were performed at pH 7.0, there was a slight diminution of activity seen with glutamate and a comparable small increase seen with glucose-6-phosphate (data not shown). Incubation of parasites isolated by saponin lysis of normal infected erythrocytes demonstrated an enrichment of glutamate-dependent GSSG reduction, but there remained a twofold to threefold excess of G6PD-derived activity. This presumably represents mostly contamination with host proteins (data not shown).

DISCUSSION

These data support the observations of Usanga and Luzzatto[4] of a parasite-derived G6PD enzyme. The parasite enzyme is much easier to detect in G6PD Mediterranean red cells, where the background enzyme activity approaches zero by spectrophotometric techniques. The amounts present are still very difficult to detect in crude lysates by spectrophotometric methods, because the presence of hemoglobin limits the concentration at which the lysates can be used. The assay used in this report does not directly depend on spectrophotometry of hemoglobin-containing samples and therefore does not suffer from this limitation. On the other hand, where a great deal of enzyme is present (as in G6PD-normal red cells), it is very difficult to detect a small increment from any parasite-derived G6PD enzyme. Therefore, our failure to detect "supernormal" activity in G6PD-dependent GSSG reduction in infected normal red cells should not be taken to mean that the parasite enzyme is not present in normal infected red cells. Techniques that physically separate the human and parasite enzyme would be better suited for the detection of a parasite G6PD in the presence of the host cell enzyme.

The present data, which were obtained from parasites not fully adapted to growth in G6PD-deficient cells (according to the procedures of Usanga and Luzzatto[4]), demonstrate that parasite G6PD is expressed to some extent and does not require a prolonged period of adaption or selection before becoming detectable. While this finding is not in agreement with the data of Usanga and Luzzatto, this disparity may merely relate to differences in the strains used. Indeed, it may well be that the single growth cycle in G6PD-deficient cells that we used before beginning the experiment may be enough to stimulate parasite enzyme production in the FCR-3 strain. Unfortunately, omission of this preexperiment growth cycle would open the possibility that any G6PD enzyme activity subsequently detected could merely be a contaminant from infected normal red cells. Further experiments that follow GSSG-reducing capacity through many cycles of parasite growth need to be performed before a clear picture of parasite adaptability is obtained.

Although at this time no one can declare what level of G6PD activity is sufficient for the parasite itself, it is clear that G6PD activity of parasitized deficient red cells is still well below that of normal cells. This implies that the parasite, which requires the host red cell for the asexual stage of its proliferation, continues to be vulnerable to oxidative challenge.

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CONCISE REPORT

Antibodies to Human T-Lymphotropic Virus Type III (HTLV-III) in Saliva of Acquired Immunodeficiency Syndrome (AIDS) Patients and in Persons at Risk for AIDS

By D.W. Archibald, L. Zon, J.E. Groopman, M.F. McLane, and M. Essex

Whole saliva samples collected from available people at risk in Boston for infection with human T-lymphotropic virus type III (HTLV-III/LAV), from late 1984 through early 1985, were analyzed for the presence of antibodies to viral proteins. Fourteen of 20 (70%) acquired immunodeficiency syndrome (AIDS) patients and 14 of 15 (93%) AIDS-related complex (ARC) patients had salivary antibodies that reacted with the virus-encoded glycoproteins gp160 and gp120 of HTLV-III infected cells. All of the AIDS and ARC patients had serum antibodies to the same antigens. Of 20 sex partners of AIDS/ARC patients, nine (45%) showed anti-HTLV-III antibodies, and four of 18 (22%) healthy homosexual males also were positive for such antibodies. Serum and salivary antibody status were the same in these groups. A minority of those patients positive for salivary antibodies to *env* gene-encoded gp160 and gp120 also had salivary antibodies to *gag* gene-encoded proteins of 55,000, 24,000, and/or 17,000 daltons. Immunoglobulin A (IgA) class antibodies comprised the majority of the salivary antibody response. The spectrum of HTLV-III proteins detected by the salivary and serum antibodies was similar. The possibility that secretory IgA from the gut-associated lymphoid system may play a role to restrict salivary transmission of HTLV-III should be considered.

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IN THE UNITED STATES and Europe, the acquired immunodeficiency syndrome (AIDS) occurs primarily in sexually active homosexual men, intravenous drug users, and blood product recipients.[1,2] Heterosexual partners of all of the above have developed AIDS.[3] The apparent routes of transmission of the human T-lymphotropic viruses (HTLV) include close sexual contact, transfusion of blood products, and parenteral exposure. One member of the HTLV family of retroviruses, HTLV-III/LAV, is generally accepted to be the etiologic agent of AIDS.[4-7] This virus has been isolated from a high percentage of patients with AIDS or AIDS-related complex (ARC),[5] and antibodies to it have been found in 88% to 100% of AIDS patients and high-risk individuals.[8,9]

Another horizontally transmissable retrovirus, feline leukemia virus (FeLV) is responsible for a series of lymphoproliferative and cytopathic diseases similar to those of the HTLV family. FeLV has been shown to be transmissable via saliva,[10] and it replicates well in the salivary gland acinar cells.[11] Moreover, saliva contains the highest level of infectious particles of any body fluid and is easily passed by mutual grooming in communally housed cats. Oronasal lymphoid cells are the first cells to show evidence of FeLV infection following an oral inoculation[11]; thus, the upper respiratory tract can also be the portal of viral entry as well as the site of transmission.

HTLV-III infectious particles have recently been isolated from the saliva of four of ten ARC patients and four of six healthy homosexuals, and retroviral particles were observed by electron microscopy in the saliva in one of four AIDS patients tested.[12] These patients were all seropositive for antibodies to HTLV-III.[12] Saliva passed via kissing has also been implicated as a vehicle of transmission in at least one case of HTLV-III infection.[13]

In respiratory viral diseases, the presence of immunoglobulin A (IgA) antibodies, which are found in higher concentrations in mucosal secretions, ie, saliva, correlates better with viral immunity than does the level of antiviral serum antibodies.[14] We report here that antibodies directed to HTLV-III proteins were found in the whole saliva of patients with AIDS and ARC.

MATERIALS AND METHODS

Whole saliva was collected from patients and healthy participants in Boston, during late 1984 and early 1985, and stored at −70 °C before being analyzed under code. Following 1:2 dilution with phosphate buffered saline or water, samples were centrifuged and passed through 0.22 μm pore size filters (Millipore Corp, Bedford, Mass). Radioimmunoprecipitation of ^{35}S-cysteine–labeled H9/HTLV-III–infected and H9-uninfected cell lysates was performed as previously described[15] except that Protein-A beads were first reacted with 10 μL of sheep anti-human secretory IgA serum (Cappel Laboratories, Inc, Cochranville, Pa) before addition of 150 μL of the saliva samples. Serum from an AIDS patient known to be positive for HTLV-III antigens p24, p55, gp120, and gp160 was used as a positive control. Immunoprecipitates were analyzed in a 10% acrylamide resolving gel with a 3.5% stacking gel, according to the discontinuous buffer systems of Laemmli[16] Autoradiography was carried out for five days at −70 °C. Serum samples from the same patients were obtained at approximately the same time and were tested by immunoprecipitation as described earlier.[15]

RESULTS

The positive saliva samples reacted with proteins of molecular weights 160,000 and 120,000 that were present in the

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infected cell lysate and not in the uninfected cell lysates. These correspond to *env* gene glycoproteins from HTLV-III as previously reported.[9,17] Salivary antibodies usually did not recognize *gag* viral proteins p55 and p24 that were precipitated by serum from an AIDS patient with known high anti–HTLV-III titers as shown in Fig 1. However, proteins of molecular weight 55,000, 38,000, 27,000, 24,000, and 17,000 were occasionally precipitated by some saliva samples that also contained antibodies to gp160 and gp120. Of 20 AIDS patients, 14 had salivary antibodies, while 14 of 15 ARC patients had salivary antibodies to gp160 and gp120. Nine of 20 healthy sex partners of AIDS and ARC patients were also salivary antibody positive. Two of these partners of AIDS patients had virus isolated from their plasma, but had no serum antibodies and also had no evidence of antibodies in their saliva.[13] Four of 18 healthy homosexual males were positive for salivary antibodies. As additional controls, ten patients hospitalized for cancer therapy, including five leukemia/lymphoma patients and five carcinoma patients, were also negative. Eleven laboratory workers with exposure to HTLV-III products were similarly negative for salivary antibodies to HTLV-III. A serum antibody positive transfusion healthy patient also had salivary antibodies to HTLV-III proteins, indicating that the gut-associated lymphoid system can be stimulated by systemic HTLV-III infection or that viral replication could occur there.

The presence of salivary antibody to HTLV-III correlated with the serum antibody status except that not all serum antibody positive AIDS patients had salivary antibody. The class of antibody in the saliva that reacted with the virus antigens was IgA, since failure to use an anti-IgA antibody to bind the salivary immunoglobulin led to a greatly diminished reactivity. The data is summarized in Table 1

DISCUSSION

Mucosal IgA is known to be protective against some viral diseases, presumably by blocking viral attachment to and/or entry into cells. FeLV is an exogenous transmissable retrovirus, as is HTLV-III, that can be passed orally via saliva even though the virus cannot survive the pH of the stomach. Therefore, the FeLV retrovirus must be able to enter the mucosal cells of the oropharyngeal region. Mucosal antibodies found in secretions such as saliva would be expected to be at least partially protective against this process. Since infectious HTLV-III has been found in saliva, the possibility exists that mucosal IgA antibodies may play a protective role against virus transmission either by preventing oropharyngeal viral entry or by blocking virus and preventing viral spread via saliva. The envelope directed IgA antibodies found in saliva would be excellent candidates for the blocking action. The oral polio vaccine protects not only by initiating systemic antibodies, but also by stimulation of IgA-producing lymphocytes in the alimentary tract. Reinfection of the gut is greatly reduced by the local IgA immunity.[18] Similar-

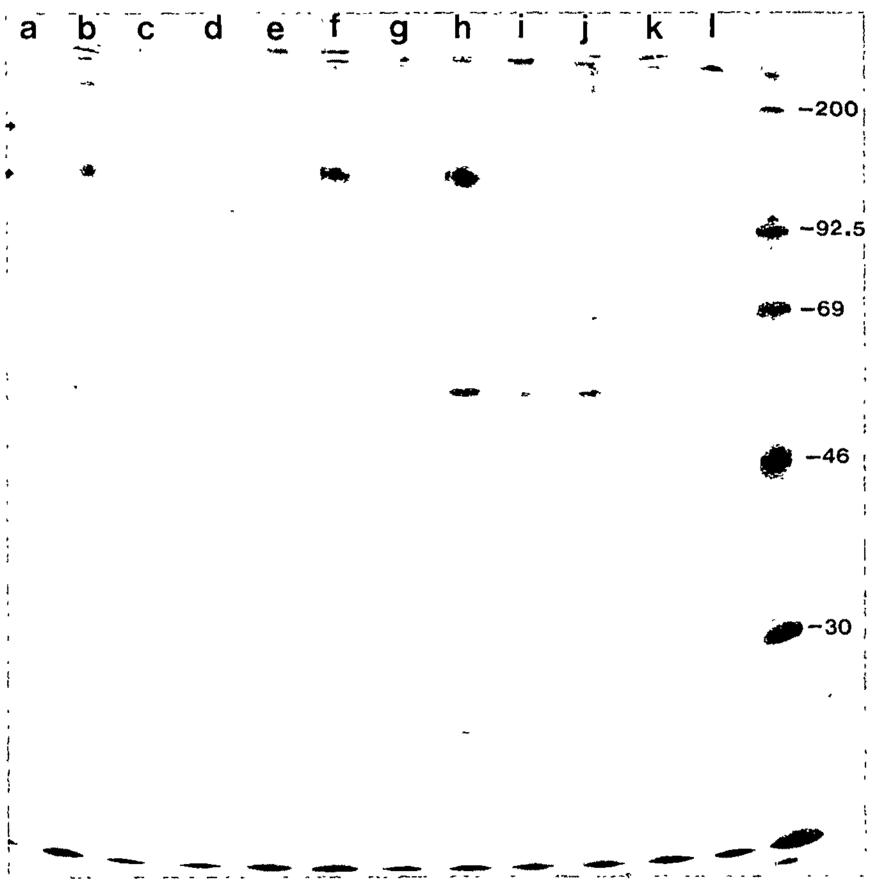

Fig 1. Soluble cell lysates were prepared from H9 (lanes a, c, e, g, i, and k) and H9/HTLV-III (lanes b, d, f, h, j, and l) cells exposed to ^{35}S-cysteine, harvested, and lysed with radioimmunoprecipitation assay (RIPA) buffer. These lysates were then reacted with 150 μL of a 1:2 dilution of saliva which had been bound to Protein A-Sepharose CL-4B (Protein A-beads, Sigma, St Louis) with 10 μL of sheep anti-human IgA sera. Samples were as follows: saliva from AIDS patient No 1 (lanes a and b); normal laboratory worker (lanes c and d); AIDS patient No. 2 (lanes e and f); AIDS patient No. 3 (lanes i and j); AIDS patient No. 4 (lanes k and l); reference serum from AIDS patient known to be positive for HTLV-III antigens (lanes g and h). Approximate molecular weights in thousands are at the right.

Table 1. Presence of Serum and Salivary Antibodies to HTLV-III

		No Positive		
Health Status	No Tested	Serum Antibodies	Salivary Antibodies to gp160/gp120	Salivary Antibodies to p55/p24
AIDS	20	20	14	2
ARC	15	14	14	4
Healthy sex partners of AIDS/ARC	20	9	9	1
Healthy homosexuals	18	4*	4	2
Cancer patients	10	NT*	0	0
Laboratory workers	11	0*	0	0
Healthy transfusion recipient	1	1	1	0

Abbreviation NT, not tested

*Sera from two laboratory workers, one healthy homosexual, and all cancer patients were unavailable for testing

ly, mucosal IgA in vaginal fluid, semen, saliva, gut secretions, and tears could be evaluated for its ability to alter HTLV-III infection.

The fact that only 70% of AIDS patients had detectable salivary antibody may be related to the low antibody titers usually found in saliva which could be reduced even more as a result of the immunodeficient state in AIDS. The demonstration of salivary antibodies in all other persons who were serum antibody positive indicates that the gut response is closely linked to the systemic humoral response. Further, it may also be related to salivary viral levels. Infectious virus in saliva has been shown to be more easily isolated from healthy homosexuals and ARC patients than from AIDS patients.[12] Thus, salivary antibody levels may drop as salivary viral levels drop. Assuming a normal concentration of 20 mg/100 mL[19] of IgA in whole saliva as compared with 328 mg/100 mL of IgA and 1,230 mg/100 mL IgG in serum, then there is only 1/80th as much potential antibody in saliva as compared with total serum levels. Salivary IgG levels are usually negligible even when transudation of serum from around the teeth is included.

Gp160 and gp120 are the likely targets of virus neutralizing antibodies. The finding of IgA antibodies directed toward HTLV-III *env* proteins in saliva also provides an opportunity for studies of IgA in other body fluids, such as semen, vaginal fluids, and tears. The role of these antibodies, in the various secretory fluids, may play in viral transmission has not been evaluated. If salivary IgA antibodies are found to be neutralizing, then questions concerning the strain specificity of antibodies directed toward HTLV-III proteins should be addressed for all mucosal fluids.

Salivary IgA antibodies were detectable in all the subjects (27 of 27) that were positive for serum antibodies except the AIDS patients. This raises the possibility that a screening test for the detection of virus-infected healthy carriers might be conducted using saliva rather than blood.

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CONCISE REPORT

Immunoglobulin Gene Rearrangements in Remission Bone Marrow Specimens From Patients With Acute Lymphoblastic Leukemia

By Barbara A. Zehnbauer, Drew M. Pardoll, Philip J. Burke, Michael L. Graham, and Bert Vogelstein

Recombinant DNA probes for the joining (J_H) segment of the immunoglobulin heavy chain gene were used to detect molecular rearrangements of this gene in the DNA of bone marrow cells obtained during remission of acute lymphoblastic leukemia (ALL). This molecular approach was optimized and found to exceed the sensitivity of conventional morphologic screening for detecting residual leukemia cells; one leukemic cell in 500 normal nucleated bone marrow cells was easily detected using this approach. In the present study, bone marrow from three of seven patients in complete clinical remission (defined morphologically) contained leukemic cells in these proportions. This analysis may be of use in evaluating the status of clinical remission in selected ALL patients.

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THE STAGES of leukemia are usually assessed by morphologic examination of cells from the peripheral circulation and/or bone marrow of patients. In general, bone marrow aspirates containing less than 5% blast cells and recovering normal elements indicate a remission stage. However, this level of detection is obviously suboptimal since patients showing less than 5% blast cells could still have millions of leukemic cells in their bone marrow. The presence of an increased normal blast population due to the effects of receiving drug treatment can artificially raise the number of normal blast cells without actual leukemic cell proliferation. Furthermore, in the L_1 and L_2 forms of acute lymphoblastic leukemia (ALL), it is not uncommon to see lymphocytic infiltration in the recovering bone marrow. Whether these cells are of normal or malignant origin is not always clear from morphologic examination.

Among the recent findings in acute lymphocytic leukemia is the rearrangement of immunoglobulin genes in the vast majority of ALL cases either of null or B cell origin.[1] One of the first events leading to expression of immunoglobulin (Ig) genes is the recombination of the distant gene segments, designated variable (V_H), diversity (D), and joining (J_H), of the Ig heavy chain gene.[2]

Intervening DNA segments are deleted during this recombination. The joined segments combine with a constant region heavy chain gene (C_H) and together code for the Ig heavy chain protein.[3] The rearrangement of Ig gene segments has been detected in leukemic cells by examining the changes in electrophoretic mobility of DNA fragments, produced by restriction endonuclease cleavage, which bridge the point at which recombination occurred. Most ALL cells contain a rearranged Ig heavy chain gene,[1] even though the light chain genes are often not rearranged.[4]

The detection of the presence of clonally derived cells with specific Ig gene rearrangements could be useful in evaluating the remission status of ALL patients.[5,6] However, to our knowledge, the utility and logistics of such a study has not been reported. We describe here our initial experience using this strategy in patients in remission. By optimizing several parameters, we noted that remission marrows containing as few as one leukemic cell in 500 normal cells could be readily identified. Furthermore, bone marrows from three of seven patients in complete remission (defined morphologically) contained leukemic cells in proportions allowing their detection by this assay. This analysis could be a useful adjunct to morphologic examination, especially in patients in whom alternative therapies are being considered or in marrows of questionable morphology.

MATERIALS AND METHODS

Cells Bone marrow aspirates were diluted 1:5 with RPMI-1640 and allowed to sediment at 1 g for one hour Cells from the buffy coat and the floating spicules (released by repeated passage of the spicules through a 25G needle) were pooled and washed with RPMI-1640. Mononuclear cells were then purified on Histopaque 1.077 (Sigma, St Louis) step gradients. A minimum of 3×10^6 purified mononuclear cells was required for this analysis of rearranged Ig genes.

Hybridization analysis DNA was purified by sodium dodecyl sulfate (SDS)-proteinase K digestion[7] followed by phenol-chloroform extraction and DNA concentrations were determined by the diphenylamine method.[8] DNA was cleaved consecutively with *Hind*III and *Bam*H1 restriction endonucleases obtained from Bethesda Research Laboratories (Bethesda, Md) using 10 U of enzyme per microgram of DNA in reaction buffers recommended by the supplier. Of the cleaved DNA, 7.5 μg were concentrated by ethanol precipitation and subjected to vertical agarose gel electrophoresis using a slot of 3 × 8 mm. Electrophoresis, transfer to nitrocellulose, and hybridization were performed as previously described[9,10] The *Bam*H1–*Hind*III fragment of the J_H region, cloned in plasmid pBR322, was provided by Dr Philip Leder. Nick translation of this plasmid clone was performed using the kit from Amersham (Arlington Heights, Ill) following the supplier's conditions but using 10 ng of DNA and 50 μCi of α^{32}P-dCTP (3,200 Ci/mmol) per reaction for one hour at 17 °C. Autoradiography was performed for 15 hours at −80 °C using Kodak XAR-5 film (Rochester, NY) and Dupont Lightning Plus intensifying screens (Dupont, Wilmington, Del).

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RESULTS

In order to optimize the detection of clonally derived cells containing identically rearranged Ig genes, we concentrated on two aspects of the analysis: methods of bone marrow preparation and nucleic acid hybridization techniques. It was of paramount importance that the bone marrow aspirate be drawn with minimal blood contamination, which could obviously dilute any leukemic cells present in the marrow with a much larger number of normal circulating cells. A two- to fivefold enrichment for mononuclear cells was achieved by techniques described in the Materials and Methods section.

Molecular biological parameters were optimized by implementing a model bone marrow population prepared by in vitro mixing of nonrearranged DNA from normal cells with varying amounts of clonally rearranged DNA from leukemic blast cells. One variable examined was the portion of the J_H region that would produce a strong "signal" upon hybridization to the rearranged DNA fragment and at the same time yield a minimum of "noise" or background hybridization to other nonspecific sequences. In this regard, several subfragments of the J_H region (Fig 1) of the Ig heavy chain gene were tested in hybridization. Fragments A through E, shown in Fig 1, which were produced by cleavage with the enzyme Cpf I (Worthington Diagnostics, Freehold, NJ), and the entire *Bam*H1–*Hind*III fragment, were tested individually as hybridization probes. Fragments A and B did not detect most rearranged genes, fragment C yielded the same background but less signal than the total plasmid clone, and fragments D and E yielded reduced background but also reduced signal Therefore, the 6 kb *Bam*H1–*Hind*III fragment encompassing all the J_H genes was used in further experiments.

Three different radiolabeling techniques of the cloned J_H *Bam*H1–*Hind*III fragment were compared. The conditions were varied to produce a probe with a high specific activity, a low degree of random hybridization or background, and a level of detection sufficient to detect <5% leukemic cells in a normal population. Synthesis of RNA from the J_H probe using the SP6 polymerase system[11] (Promega Biotec, Madison, Wisc) fulfilled the first two criteria but not the third. "Oligo-labeling," performed using random hexamers as primers,[12] as well as nick translation produced probes of high sensitivity, but the background was somewhat less using the nick translated probe. A high specific activity (2.5×10^9 dpm/μg) using nick translation was obtained by using only 10 ng of the J_H plasmid in a reaction mixture containing 50 μCi of α^{32}P-dCTP (3,200 Ci/mmol)

The choice of restriction endonuclease with which the genomic DNA was cleaved was varied to select a combination which would reveal rearrangement of J_H sequences in a DNA fragment with a significantly different mobility from the normal nonrearranged DNA upon electrophoresis. A combination of *Bam*H1 plus *Hind*III produced a 6 kb DNA fragment in a normal sample (nonrearranged DNA) corresponding to the complete J_H probe while rearranged fragments in the leukemic samples were usually of significantly different size. Other enzyme combinations tested did not produce easily distinguishable, rearranged fragments from leukemic cells as frequently as the *Bam*H1–*Hind*III combination. In addition, the *Bam*H1–*Hind*III combination had optimal complementarity with the probe. Fig 2A shows a typical experiment in which rearranged DNA fragments were detected in DNA extracted from leukemic cells of patients during the active phase of their disease (presentation or relapse). In each case, one to three rearranged fragments were identified and usually a normal, 6 kb fragment was also observed, representing unrearranged Ig alleles still remaining in the leukemic cells or nonleukemic cells present in the cell fractions used for analysis.

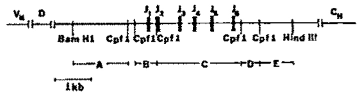

Fig 1. Map of immunoglobulin J_H region of the human genome. CPF-1 sites were mapped in the region described in ref. 3.

Using the optimized methods, mixing experiments were performed to demonstrate the limits of detection of leukemic cells in the assay. One leukemic cell in 333 normal cells could be clearly detected in the original autoradiograph (Fig 2B). The source of the normal cells (placenta or peripheral blood mononuclear cells) did not affect this sensitivity. Since the cell fractionation procedure resulted in a two- to fivefold enrichment of mononuclear cells from bone marrow, one could specifically detect, at minimum, one leukemic cell in 500 normal bone marrow cells.

We next examined actual remission bone marrow samples to determine how frequently leukemic cells could be detected using the recombinant DNA analysis In each case, the bone marrow sample examined had been independently diagnosed as indicating complete clinical remission by morphologic methods. In Fig 3, patient No. 1 is illustrative of the results that can be achieved. This patient was a 25-year-old male who presented with ALL in Feb 1985 (Table 1). Clinical remission was achieved in May 1985 following treatment with prednisone and vincristine and then arabinoside-c (ara-c) with daunomycin given in a timed sequence. At the time of bone marrow recovery, an aspirate of the bone marrow was obtained. Maturation of all cell types was observed with normal numbers of blast cells (<5%) After fractionating the bone marrow to enrich for mononuclear cells, we analyzed the DNA of the cells for rearranged bands characteristic of the patient's leukemic cells. In DNA from his leukemic cells (obtained at the time of initial disease presentation), two rearranged fragments of 3.5 kb and 2.5 kb were detected (Fig 3, lane L, patient No. 1). These same fragments were detected in the DNA from the clinical remission bone marrow (Fig 3, lane R, patient No 1). Note that the relative intensity of the rearranged bands is much less in lane R than in lane L, even though lane R contained 7.5 μg of DNA and lane L contained only 0.5 μg of DNA. We estimated that the rearranged bands in the remission sample corresponded to 1% to 2% leukemic cells in this bone marrow. The prevalent band at 6 kb derives from normal cells in the bone marrow The 4.3 kb fragment was an internal control for the hybridization reaction.

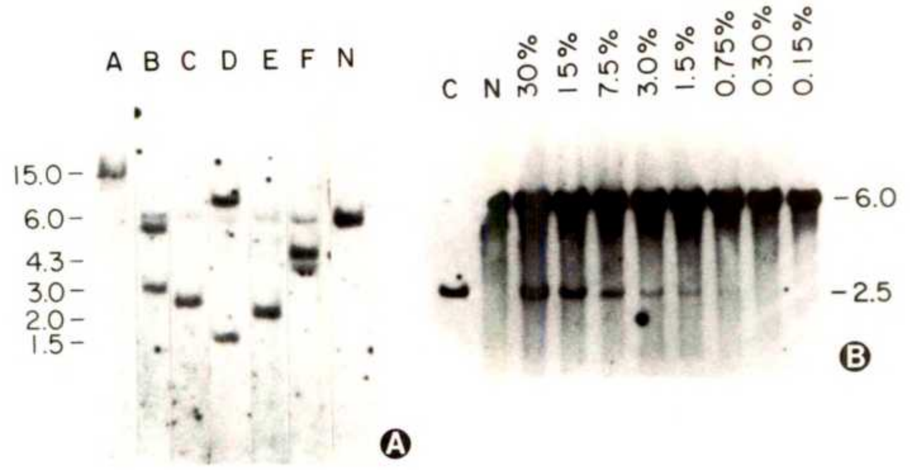

Fig 2. (A) Rearranged J_H fragments in patients with leukemia. DNA was extracted from ALL cells of six patients (A through F) during the active phase of their disease (presentation or relapse). As described in Materials and Methods, 0.5 to 2.0 μg of DNA was cleaved with *Bam*H1 and *Hind*III, and these digests were used in electrophoresis, Southern transfer, and hybridization. An autoradiograph of the Southern blot is shown with sizes of fragments noted on the left in kilobases (kb). Patients A, C, and E had one rearranged allele and one deleted allele, while patients B, D, and F had patterns which indicated a different rearrangement for each of two alleles. (B) Mixing experiment as model of remission bone marrow. Placenta DNA and DNA from patient C in Fig 2A was digested with *Bam*H1 and *Hind*III. The normal DNA was mixed with leukemic DNA at the indicated ratios representing 7.5 μg normal plus 2.25 μg leukemic (1:3.3 or 30%), 7.5 μg normal plus 1.12 μg leukemic (1.0:6.7 or 15%), 7.5 μg normal plus 0.563 μg leukemic (1:13.3 or 7.5%), 7.5 μg normal plus 0.225 μg leukemic (1:33 or 3%), 7.5 μg normal plus 0.113 μg leukemic (1:66 or 1.5%), 7.5 μg normal plus 56.3 ng leukemic (1:133 or 0.75%), 7.5 μg normal plus 22.5 ng leukemic (1:333 or 0.3%), and 7.5 μg normal plus 11.3 ng leukemic (1:666 or 0.15%), respectively. Also individually represented (lanes C and N, respectively) are 2.25 micrograms of leukemic cell DNA and 7.5 μg of normal DNA.

Similar analyses were performed on a total of seven patients (Table 1) in whom morphologically normal clinical remission bone marrows were obtained. Of these seven patients, three exhibited leukemic cells in the remission marrows assessed by the recombinant DNA method (ie, patients No. 1, 2, and 3 of Fig 3 and Table 1). Two controls were required for unambiguous interpretation of these results. First, DNA from the leukemic cells of the same patients was used as a positive control to ensure that the rearranged fragments observed in the remission samples corresponded to the fragments detected in the leukemic cells. Second, normal DNA from the peripheral blood granulocytes of the patient was included as a negative control to verify that any faint rearranged bands were not attributable to the patient's normal genes. In Fig 3, patients No. 2 and 3 displayed rearranged fragments in their remission bone marrow samples (noted with asterisks in lanes R, R_1, and R_2) which were not present in the DNA from normal cells of these patients (lane G). In the R_2 remission sample of patient No. 3, a third rearranged band was present that was not observed in the original leukemic cells or the granulocytes from this patient. This third fragment may have represented a further rearrangement of the immunoglobulin gene which occurred in a subfraction of the leukemic cells.[13] In patients No. 2 and 3, the number of leukemic cells in the remission specimens was estimated as one in 500 and one in 200 nucleated marrow cells, respectively. Patient No. 4 is included to illustrate a clinical remission bone marrow sample with less than one leukemic cell in 500 normal cells as detected in our assay.

DISCUSSION

Using recombinant DNA techniques described above, we have detected one leukemic cell among more than 500

Fig 3. Rearranged DNA fragments of leukemic blasts in remission bone marrows of ALL patients. Each panel depicts a different patient's samples. L denotes DNA from leukemic cells, either presentation or relapse. R denotes remission as defined by morphologic examination (<5% blast cells). Patient No. 3 had two separate remission episodes analyzed. G denotes DNA from the granulocytic cells (included as a negative control for DNA rearrangements). All lanes depict 7.5 μg of DNA digests except patient No. 1, lane L (0.5 μg), and patients No. 3 and 4, lane L (1 μg). Size is indicated at left in kilobases. All samples contained 1 pg pBR322 DNA as a control for hybridization and transfer efficiency. Dots indicate rearranged J_H fragments present in the various samples.

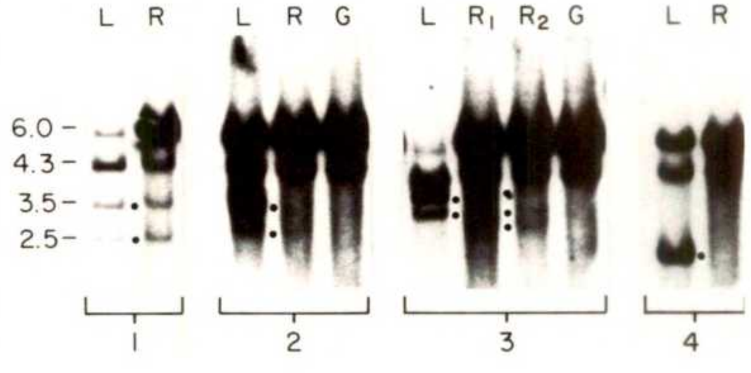

Table 1. Characteristics of ALL Cases Studied for Ig Gene Rearrangement

Patient No.	ALL Subclassification FAB	ALL Subclassification surface marker	Age	Date of Presentation	Date of Relapse	Date of Remission Marrow Examined
1	L2	Ia+, CALLA+, Leu12+, Leu14+, OKB2+	25	2/6/85	None	5/16/85
2	L1?	Ia+, CALLA+, Leu12+	5	3/15/83	9/28/84	12/5/84
3	L2	Ia+, Leu12+	36	11/17/84	None	1/8/85 (R1) and 3/21/85 (R2)
4	L1	Ia+, CALLA+, Leu12+	11	10/17/84	None	11/12/84
5	L2	Ia+, CALLA+, B1+	8	1/1/85	None	1/30/85
6	L2	Ia+, CALLA+	13	7/20/83	1/11/85	5/13/85
7	L3	Ia+, B1+	40	1/19/85	None	3/28/85

Abbreviation: FAB, French-American-British.

normal nucleated cells in remission bone marrows. In order for this technique to be useful in the analysis of remission bone marrows, a significant number of bone marrows must contain enough leukemic cells to be recognized by the recombinant DNA assay (more than one in 500) but less leukemic cells than sufficient for recognition by morphologic evaluation (1/20). In the initial analyses of the seven patients reported here, three of the bone marrow samples exhibited numbers of leukemic cells in this range. Obviously, further studies are required to define the clinical situations in which this type of analysis will be most useful. The significance of the presence of low numbers of leukemic cells to the clinical status of the patient also remains to be determined. However, for the present, this analysis would appear to be helpful in diagnosis of patients with questionable remission status as assessed morphologically and in patients in whom alternative therapy, such as autologous or allogeneic bone marrow transplantation, is being considered.

ACKNOWLEDGMENT

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CONCISE REPORT

C-*sis* and C-*abl* Expression in Chronic Myelogenous Leukemia and Other Hematologic Malignancies

By Paolo Romero, Mark Blick, Moshe Talpaz, Edwin Murphy, Jeane Hester, and Jordan Gutterman

Cellular oncogenes have been localized at the breakpoints of characteristic chromosomal rearrangements occurring in certain hematologic malignancies. This has been reported to result in aberrant expression of the involved oncogenes. Over 90% of chronic myelogenous leukemia (CML) is characterized by a reciprocal translocation that brings c-*abl* from chromosome 9 to chromosome 22, and c-*sis* from chromosome 22 to chromosome 9. To investigate the possible role of these two oncogenes in the leukemic process, we studied their expression in a number of fresh samples obtained from patients with various forms of leukemia, by Northern blot analysis using c-*onc* probes. Seven of 24 samples obtained from patients with either CML or chronic myelomonocytic leukemia expressed a normal 4.0-kilobase (kb) c-*sis* transcript. C-*sis* expression was found only in the accelerated/blast phases but not in the chronic phase of CML. All of the CML Philadelphia chromosome-positive (Ph^{1+}) samples expressed an aberrant 8-kb c-*abl* transcript. The expression of c-*sis* in hematopoietic malignancies constitutes a novel finding.

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IN MORE THAN 90% of the cases of chronic myelogenous leukemia (CML), a characteristic chromosomal abnormality, the Philadelphia (Ph^1) chromosome, is present.[1] This Ph^1 chromosome consists of a shortened chromosome 22 that results from a balanced translocation involving chromosome 9 and 22[2] [t(9;22)(q34;q11)]. The oncogene c-*abl*, normally located on chromosome 9, band q34, near the 9:22 translocation breakpoint, is translocated to chromosome 22.[3] C-*sis*, normally located on chromosome region 22q12.3-q13.1 appears to be at a significant distance from the 9:22 translocation breakpoint and is reciprocally translocated to chromosome 9.[4]

To evaluate the possible role of the c-*abl* and c-*sis* oncogenes in the leukemic process, we have analyzed their expression in a number of hematopoietic cells. We report that a normal size 4-kilobase (kb) c-*sis* transcript is expressed in five of 20 fresh CML samples and in two of four fresh chronic myelomonocytic leukemia (CMML) samples. We have also analyzed these same samples for c-*abl* expression and confirmed that an aberrant 8-kb mRNA species[5,6] is present in all CML Ph^{1+} samples, but not in the CMML samples nor in the samples from other leukemias. The 8-kb c-*abl* mRNA is present in all clinical phases of CML.

MATERIALS AND METHODS

Samples were obtained by leukapheresis. RNA was prepared using the guanidinium/cesium chloride method.[7] Polyadenylated RNA was selected by chromatography on oligo(dT)-cellulose columns. The polyadenylated RNA (5 μg/lane) was size fractionated by electrophoresis in 1.1% agarose gels containing 2.2 mol/L formaldehyde, transferred to nitrocellulose filters with 20× SSC (SSC = 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate), and hybridized to radiolabeled ^{32}P-DNA probes at 42 °C for 12 hours by a modification of the method described by Thomas.[8] The probes were radiolabeled by nick translation to a specific activity of 1 to 3×10^8 cpm/μg of DNA. The filters were then washed in 0.1× SSC and 0.1% sodium lauryl sulphate at 60 °C for 60 minutes, air dried, and autoradiographed with intensifying screens for two to seven days at −70 °C.

For DNA analysis, restriction endonuclease digestion of high molecular weight DNA was performed as recommended by the supplier, and blot hybridization was carried out as described by Southern.[9] For our *sis* hybridization experiments, we employed an *Eco*R1/*Pst*1 fragment of clone L33,[10] corresponding to the 5′ region of c-*sis* and a *Bam*HI fragment of clone L33 corresponding to the 3′ end of c-*sis*. The probes for our *abl* hybridization experiments were derived from subclones of c-*abl* containing cosmids. We utilized an *Eco*R1/*Bam*HI fragment corresponding to the most 5′ v-*abl* hybridizing region and a *Hind*III/*Eco*R1 fragment corresponding to the 3′ region of c-*abl*.[3] In the Northern experiments, only the 3′ c-*sis* probe and the 5′ c-*abl* probe were used. In the Southern experiments, all four probes were used.

RESULTS

As shown in Fig 1 and Table 1, we detected a 4-kb c-*sis* transcript in three samples obtained from patients in the accelerated phase (AP) of CML (CML-AP), in two samples obtained from patients in the lymphoid CML blast crisis (BC) phase (CML-BC), and in the K562 cell line, derived from a patient with CML-BC. C-*sis* expression was of low intensity and comparable with the level detected in the Hut 102 cell line, derived from a patient with cutaneous T cell lymphoma and known to express c-*sis*.[11] We also found c-*sis* expressed in two samples from patients with CMML, a disorder frequently confused with CML, and often called Ph^1-negative (Ph^{1-}) CML. With the exception of one patient with CML-BC, all the samples that expressed the 4-kb c-*sis* transcript also expressed a less intense 3-kb species as reported by others (Fig 1).[12] An analysis of the DNA using the restriction enzymes *Eco*RI and *Bgl*II and probes from both ends of the c-*sis* gene disclosed no DNA amplifications or rearrangements in those samples that expressed c-*sis* (data not shown).

Fig 2 and Table 1 show our results using the c-*abl* probe. An aberrant 8-kb transcript was expressed in all Ph^{1+} CML

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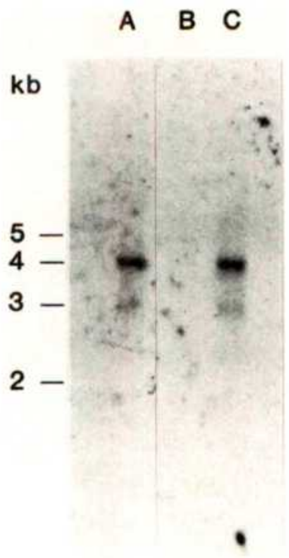

Fig 1. Northern blot analysis using a c-*sis* probe demonstrating c-*sis* expression in fresh CML-AP (lane A) and in the control Hut 102 cell line (lane C). C-*sis* is not expressed in a fresh CML-CP (lane B). 5-kb corresponds to 28s rRNA and 2-kb to 18s rRNA, which were used as molecular size markers.

samples. This 8-kb c-*abl* transcript was not detected in fresh samples obtained from patients with Ph^{1-} diseases: CMML, chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML), and the Hut 102 cell line. The two normal c-*abl* transcripts of 7- and 6-kb were present in both Ph^{1+} and Ph^{1-} samples with the exception of two samples obtained from patients with CML in blast crisis. The 8-kb transcript was the predominant c-*abl*–related mRNA species in those samples expressing it. In four of six samples obtained from patients with CML-BC, the expression of this abnormal 8-kb transcript was increased to levels comparable with those in the K562 cell line, known to have an amplified c-*abl* gene.[13] Analysis of the DNA from these fresh samples, using the restriction enzymes *Eco*RI and *Bgl*II and probes from both the 5′ and 3′ ends of the c-*abl* gene disclosed no c-*abl* amplifications or rearrangements (data not shown).

Table 1. c-*sis* and c-*abl* Oncogene Expression in CML and Other Leukemias

			No. Positive		
			c-*sis*	c-*abl*	
Cell Types	Ph^1	No. Studied	4-kb*	8-kb*	6- and 7-kb*
Fresh Cells					
CML-CP	+	6	0	6	6
CML-AP	+	8	3	8	8
CML-BC (myeloid)	+	2	0	2	1
CML-BC (lymphoid)	+	4	2	4	3
CMML	−	4	2	0	4
CLL	−	1	0	0	1
AML	−	2	0	0	2
Cell lines					
Hut 102	−	1	1	0	1
K562	+	1	1	1	1

Abbreviations: Ph^1, Philadelphia chromosome; CML-CP, chronic myelogenous leukemia-chronic phase; CML-AP, chronic myelogenous leukemia-accelerated phase; CML-BC, chronic myelogenous leukemia-blast crisis; CMML, chronic myelomonocytic leukemia; CLL, chronic lymphocytic leukemia; AML, acute myelogenous leukemia.

*Transcript size.

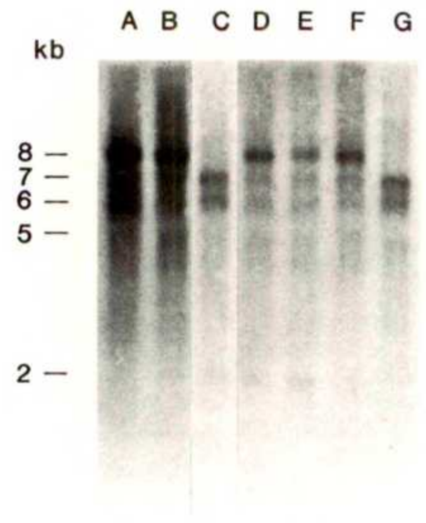

Fig 2. Northern blot analysis demonstrating an aberrant 8-kb c-*abl* transcript in the K562 cell line (lane A), a fresh CML-BC (lane B), CML-AP (lane D), two fresh CML-CP (lanes E and F). A 6- and 7-kb c-*abl* transcript is present in all samples including a fresh CMML (lane C) and the Hut 102 cell line (lane G). 5-kb corresponds to 28s rRNA and 2-kb to 18s rRNA, which were used as molecular size markers.

DISCUSSION

C-*sis*, the cellular homologue of the simian sarcoma virus oncogene, is the structural gene for the B chain of platelet-derived growth factor (PDGF),[14,15] the major mitogen of human serum. C-*sis* expression has been found in osteosarcomas, fibrosarcomas, and gliomas, but not in hematologic malignancies with the exception of the cell line Hut 102.[11,12] The mitogenic properties of PDGF may be involved in tumor development, according to an autocrine model in which the neoplastic cells proliferation is driven by growth factors produced by the tumor itself.[16] Since PDGF is reported to have mitogenic effects on hematopoietic cells[17] as well as hematopoietic microenvironment cells[18] when exogenously administered, perhaps the product of c-*sis* in CML is secreted and does have a direct dysregulatory effect on hematopoiesis. Alternatively, the product of c-*sis* in CML is not secreted and the neoplastic cells are rendered mitogenically competent by PDGF's effects on internal cellular structures as suggested by others studying an osteosarcoma cell line.[19] In any event, the role of c-*sis* in CML and CMML remains speculative at present, and its expression may be a consequence of the neoplastic state, rather than being a causal factor. The fact that c-*sis* is expressed in Ph^{1-} disease suggests that its expression is not a consequence of the 9:22 translocation. It is also of note that c-*sis* expression was found only in the accelerated or blast phases, but not in the chronic phase of CML (Table 1), raising the possibility that it may have a role in the progression of the disease.

The finding of an aberrant 8-kb c-*abl* transcript in Ph^{1+} CML has been reported previously.[5,6] We have extended these observations to include more patient samples and report that the 8-kb aberrant transcript is present in all patients and in all phases of the disease. Other investigators have recently demonstrated in the K562 cell line and fresh CML samples the presence of an abnormal c-*abl* protein with tyrosine kinase activity that is not present in normal cells.[20] It is conceivable that this protein is encoded by the 8-kb c-*abl* transcript. The nature of the 8-kb mRNA has been recently clarified by cDNA cloning.[21] It appears to be a fused transcript of the *bcr* gene of chromosome 22 and of the translocated c-*abl* gene. Our study confirms the uniform size of the aberrant transcript in spite of variable breakpoints on both chromosomes.[22] This suggests that the splicing of the large primary transcript must occur at common donor-acceptor sites.

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CONCISE REPORT

Monoclonal Antibody 12-8 Recognizes a 115-kd Molecule Present on Both Unipotent and Multipotent Hematopoietic Colony-Forming Cells and Their Precursors

By Robert G. Andrews, Jack W. Singer, and Irwin D. Bernstein

A monoclonal antibody, 12-8, prepared against KG-1a cells, recognizes an approximately 115-kd cell surface antigen and reacts with 3% to 4% of bone marrow cells, including most of the blast cells. The antigen is not expressed on peripheral blood cells. Marrow cells expressing 12-8 collected by fluorescence-activated cell sorting contained nearly all of the unipotent (CFU-GM, BFU-E) and multipotent (CFU-MIX) colony-forming cells. The isolated 12-8 positive marrow population also contained precursors of these colony-forming cells. In a two-stage long-term marrow culture system employing irradiated allogeneic marrow adherent cells, 12-8 positive cells produced both unipotent and multipotent colony-forming cells for ten weeks. Moreover, the output of colony forming cells substantially exceeded the input. Antibody 12-8 appears useful for analysis and possibly enrichment of hematopoietic progenitor cells that include colony-forming cell precursors.

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NUMEROUS monoclonal antibodies which recognize antigens expressed by normal myeloid cells of different lineages and at specific stages of maturation have now been prepared.[1,2] These antibodies are potentially useful for separating myeloid cells at distinct stages of differentiation and progenitor cells at different levels of commitment.

In the present article, we describe an antibody, 12-8, which recognizes an antigen of approximately 115 kilodaltons (kd) and reacts with a minor subset of bone marrow cells containing virtually all unipotent and multipotent colony-forming cells. Of significance is the observation that this minor population also contains cells that appear to give rise to colony-forming cells in a long-term bone marrow culture system.[3]

MATERIALS AND METHODS

Production of antibody 12-8. Seven-week-old Balb/c mice were immunized intraperitoneally with 3×10^6 KG-1a cells on days 9 and 28, and with 3×10^6 cells intravenously on day 42. On day 45, spleen cells were fused with SP2/0 myeloma cells using previously described techniques.[4] Hybrids secreting antibody that bound to KG-1a but not to peripheral blood granulocytes, in a ^{125}I-staphylococcal protein-A binding assay,[5] were formally cloned.[4] Selected clones were expanded and unfractionated culture supernatants were obtained for further studies. Antibody 12-8 was determined to be an IgM molecule using described immunodiffusion techniques[4] and was cytotoxic for KG-1a cells in the presence of complement.[4]

Peripheral blood and bone marrow cells. Samples of peripheral blood and bone marrow were obtained from normal healthy volunteers following informed consent. Peripheral blood lymphocytes, monocytes, granulocytes, red cells, and platelets were isolated as previously described.[4] The lymphocyte populations contained approximately 5% B cells and 82% T cells as determined by reactivity with anti-T cell (10.2, CD-5)[1] and anti-B cell (1F5, CD-20)[2] antibodies, and the monocytes were 65% reactive with an anti-monocyte (5F1, CDw14)[1,4] antibody. Samples of thymocytes were kindly provided by Dr P. Martin, Fred Hutchinson Cancer Research Center, Seattle.

Immunofluorescence assays and cell sorting. Reactivity of 12-8 with different cell types was determined by indirect immunofluorescence, and stained and unstained cells were separated by fluorescence activated cell sorting (FACS) as previously described.[6]

Hematopoietic progenitor assays. Unipotential colony-forming cells (CFC), BFU-E and CFU-GM, and multipotent CFCs, CFU-MIX, were cultured as previously described.[6,7] Long-term cultures were performed using Ambitube (Miles Scientific, Naperville, Ill) culture tubes with established confluent marrow adherent cell layers

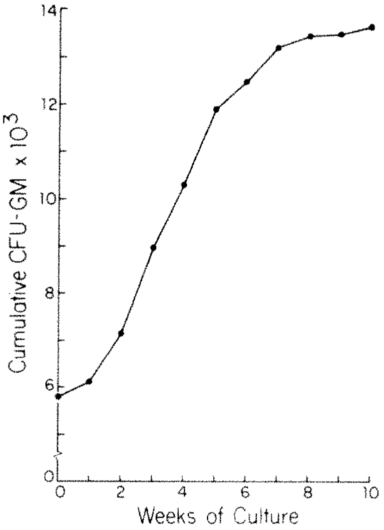

Fig 1. Cultures established with 1.2×10^5 12-8 positive marrow cells containing 5,789 CFCs were assayed weekly for the presence of CFU-GM in culture supernatants. Total CFU-GM present each week equaled CFU-GM/volume removed/(volume removed/volume in culture). Cumulative CFU-GM equals the sum of weekly mean total CFU-GM over 10 weeks of culture.

From the program in Pediatric Oncology, the Fred Hutchinson Cancer Research Center, Veteran's Administration Hospital, Seattle, and Departments of Pediatrics and Medicine, University of Washington, Seattle.

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Table 1. Reactivity of 12-8 With Normal Bone Marrow Cells Determined by Indirect Immunofluorescent Staining and Fluorescence Activated Cell Sorting

	Experiment 1			Experiment 2		
Cell Type	12-8 Positive	12-8 Negative	Unseparated	12-8 Positive	12-8 Negative	Unseparated
Blast	33	1	4	28	2	3
Promyelocyte	17	0	4	3	3	2
Myelocyte	1	6	1	3	4	7
Metamyelocyte	2	25	29	3	27	29
Band + PMN	7	37	35	4	39	40
Monocyte	13	3	5	31	4	7
Monoblast	8	1	1	11	2	1
Lymphocyte	4	1	3	5	11	5
NRBC	6	23	21	4	7	6
Other	10	2	2	7	2	1

Abbreviations: PMN, ...; NRBC, ...

Cell type data are percent of type in each population based on analysis of >250 cells on Wright-Giemsa-stained cytocentrifuge preparations.

that have been irradiated (1,148 ± 209 cGy) as previously described.[3]

Radioimmunoprecipitation. KG-1a cells (2 × 10^7) were surface labeled with ^{125}I by the iodogen method.[8] They were lysed with 0.5% NP-40 in the presence of protease inhibitors, and immunoprecipitated using Sepharose 4B (Pharmacia, Piscataway, NJ) coupled rabbit antibodies to mouse immunoglobulin,[9] and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS).[10] The control antibody, K32, which recognizes the transferrin receptor, was prepared in our laboratory (Fig 1).

RESULTS

Reaction of antibody 12-8 with hematopoietic cells. Antibody 12-8 did not react with peripheral blood lymphocytes, monocytes, granulocytes, platelets, RBCs, or thymocytes in indirect immunofluorescent assays (data not shown). However, a minor portion of normal bone marrow cells were stained. In two experiments, positively and negatively stained marrow cells were separated by fluorescence-activated cell sorting (FACS). The sorting windows for the 12-8 positive population contained 3.5% to 4.0% of analyzed cells, and the isolated 12-8 stained population was highly enriched for the least mature (blast) cells present in marrow (Table 1).

Reaction of antibody 12-8 with hematopoietic progenitors. Marrow cells that were stained or unstained by antibody 12-8 were separated by FACS and analyzed for their ability to form colonies in vitro (Table 2; day 0, Experiments 1 and 2). Virtually all of the sorted colony-forming cells were contained in the 12-8 stained population and accounted for the majority of all colony-forming cells, both unipotent and multipotent, present in unseparated marrow.

Separated cells were also cultured in a long-term culture system. Because of the paucity of isolated 12-8 bearing cells and the possibility that this population lacked accessory cells required for establishment of long-term cultures, each of the separated as well as the unseparated cell populations were placed over previously established irradiated allogeneic marrow adherent cell layers[3] (Table 2). Supernatants from

Table 2. Suspension Cultures of 12-8 Positive and 12-8 Negative Marrow Cells Isolated by Fluorescence Activated Cell Sorting

	Colonies/10^5 Starting Cells/Tube								
	12-8 Positive			12-8 Negative			Unseparated		
	CFU-GM	BFU-E	CFU-Mix	CFU-GM	BFU-E	CFU-Mix	CFU-GM	BFU-E	CFU-Mix
Experiment 1									
Day 0*	4,824 ± 296	2,487 ± 126	56 ± 23	19 ± 5	25 ± 7	0.4 ± 0.4	174 ± 6	194 ± 10	2 ± 1
Day 14	1,000 ± 143	60 ± 4	74 ± 6	0.6 ± 0.01	C	C	5 ± 1	C	C
Day 21	2,103 ± 462	103 ± 28	37 ± 7	1 ± 0.6	0.02 ± 0.01	0 ± 0	11 ± 2	1 ± 1	0.02 ± 0.01
Day 28	1,107†	33†	10†	0.8†	0†	0†	7 ± 1	0.02 ± 0.01	0 ± 0
Experiment 2									
Day 0*	4,540 ± 241	3,173 ± 385	72 ± 11	3 ± 1	6 ± 1	0 ± 0	45 ± 7	43 ± 3	0.5 ± 0.3
Day 14	282 ± 30	247 ± 92	17 ± 5	0 ± 0	0+	0+	10 ± 2	0.5 ± 0.3	0 ± 0
Day 21	183 ± 30	3 ± 2	10 ± 6	ND	ND	ND	8 ± 1	0.6 ± 0.5	0.5 ± 0.2
Day 28	205 ± 76	3 ± 2	0.8 ± 0.8	0.07 ± 0.03	0.1 ± 0.05	0 ± 0	4 ± 0.5	0.1 ± 0.1	0.2 ± 0.1

Abbreviations: C, culture plates contaminated; ND, not determined.

*Shown in boxes, day 0 (experiments 1 and 2) are the mean ± SEM number of colony-forming cells of each type detectable in replicate cultures in semisolid medium. On days 14, 21, and 28 below, the number of colony-forming cells obtained from suspension culture supernatants is shown. In experiment 1, suspension cultures were established with either 1.2 × 10^5 12-8 positive cells; 2.0 × 10^6 12-8 negative cells; or 2.0 × 10^6 unseparated cells per tube. The 12-8 positive sorting window included 3.5% of cells and the 12-8 negative sorting window encompassed 95% of marrow cells analyzed. In experiment 2, cultures were established with 1.25 × 10^5 12-8 positive cells; 3 × 10^6 12-8 negative cells; or 3.2 × 10^6 unseparate cells per tube. The 12-8 positive sorting window included 4.0% of cells and the negative sorting window included 94% of marrow cells analyzed.

†Results of cultures from a single tube.

cultures of isolated 12-8 positive marrow cells produced, over a 4-week period, a greater number of colony-forming cells than did supernatants from cultures of either unseparated or 12-8 negative cells. This was despite the fact that the cultures of 12-8 positive cells were initiated with a significantly smaller number (approximately 5%) of cells than used to initiate the other cultures. In addition, the 12-8 positive cells produced CFU-GM over a 10-week period. The cumulative number of CFU-GM produced by a single 12-8 long-term culture initiated with 1.25×10^5 cells over this period shown in Fig 1 demonstrates the total output of the culture substantially exceeded the input of colony-forming cells (input: 5,789; output: 14,354). Not shown is the growth of BFU-E (25, 25 and 0 colonies) and CFU-MIX (8, 0, and 8) from cells harvested at weeks 8, 9, and 10, respectively.

Biochemistry. The antigen recognized by the antibody 12-8 has a molecular weight of approximately 115 kd under reducing and nonreducing conditions (Fig 2) as demonstrated by radioimmunoprecipitation from ^{125}I surface-labeled KG-1a cells.

DISCUSSION

This article demonstrates that the myeloid differentiation antigen recognized by the 12-8 antibody is expressed by hematopoietic CFCs and their precursors detectable in long-term marrow culture. The presence of 12-8 antigen on precursors of CFCs is suggested by the ability of 12-8 positive cells to give rise to CFCs over an extended period of time with a substantially larger output of CFCs than were present when the cultures were started.

On the basis of cellular distribution and molecular weight, the 12-8 antigen may be the same as that identified by Civin et al[11] and Tindle et al.[12,13] These authors described antibodies MY-10 and B1-3C5, respectively, that recognized molecules with approximate molecular weights of 115 to 120 kd present on a minor population of immature hematopoietic cells, including CFU-GM, BFU-E, and CFU-MIX. Few other differentiation antigens have been described which are present on both unipotent and multipotent hematopoietic progenitors. Examples include an antigen identified by L4F3 and an antibody of similar specificity, MY-9[14]; an antigen recognized by RFB-1[15]; as well as HLA-DR antigens.[16] In contrast, the precursors of colony-forming cells lack the L4F3 antigen and may express some[17] but not all class II antigens,[18,19] while the expression of other antigens has not yet been evaluated. Most important, the present studies demonstrate the presence of the 12-8 antigen on the precursors of colony-forming cells as well as their colony-forming progeny.

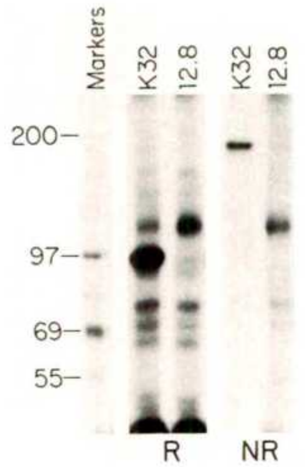

Fig 2. Immunoprecipitation by antibody 12-8 of a 115-kd antigen from surface ^{125}I-labeled KG-1a. The antibodies used were 12-8, lane 3 and lane 5; and control antibody K32 against the transferrin receptor, lanes 2 and 4. The immunoprecipitation samples were either reduced (lanes 2 and 3) or not reduced (lanes 4 and 5) and electrophoresed on 7.5% SDS polyacrylamide gel. In lane 1, ^{14}C-labeled protein markers are shown.

Although the function of the antigen recognized by 12-8 remains to be determined, this antibody appears useful for analysis of early hematopoietic development. By combining negative selection methods, eg, using antibody L4F3 and complement, and positive selection with 12-8, it now may be possible to highly enrich, if not purify, primitive colony-forming cell precursors in order to examine the conditions which lead to their proliferation and/or differentiation.

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CORRESPONDENCE

NEONATAL ALLOIMMUNE THROMBOCYTOPENIC PURPURA AND CONGENITAL PORENCEPHALY ASSOCIATED WITH A NEW MATERNAL ANTIPLATELET ANTIBODY

To the Editor.

I have read with interest the paper of Friedman and Aster entitled "Neonatal Alloimmune Thrombocytopenic Purpura and Congenital Porencephaly in Two Siblings Associated With a 'New' Maternal Antiplatet Antibody" in the June issue of *Blood*[1]

The mother's serum most likely had antibodies to platelet glycoproteins (GP) IIb/IIIa since her serum failed only to react with platelets from patient with type I Glanzmann's thrombasthenia.[2] It has been shown that autoantibodies to GP IIb/IIIa could be present in some ITP cases, especially chronic ones[3] If her serum would also react with the platelets of her oldest son, whose father was different from her present husband, this would most likely indicate that her antibodies were not specific to a "new" platelet antigen but were to GP IIb/IIIa, which is found some ITP cases.

Since we have shown that antiplatelet antibodies were persistent with short platelet survival in ITP patients in remission,[4] I would suggest that her antibodies were reminiscent from her asymptomatic ITP, causing problems related to thrombocytopenia in her fetuses.

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To the Editor·

Dr Ozsoylu suggests that serum Pen from the mother of two infants with neonatal alloimmune thrombocytopenic purpura which we describe in our recent paper[1] is an autoantibody related to asymptomatic maternal ITP. We believe that this interpretation is unlikely to be correct for several reasons. First, as stated in our report, Mrs Pen did not ever have thrombocytopenia, purpura, or bleeding problems. Second, her serum did not react with her own platelets and thus did not contain an antiplatelet autoantibody. Mrs Pen's serum also does not react with platelets from her normal sister or with platelets from an unrelated Latin American woman with posttransfusion purpura whom we have studied more recently. The data support our conclusion that serum Pen recognizes a previously undescribed high-frequency platelet alloantigen.

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ANNOUNCEMENTS

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Special Guest & Speaker: Eugene A. Stead, M.D.
For further information contact: Craig S. Kitchens, PO Box 2715, Gainesville, FL 32601

REGISTRY OF CLINICAL TRIALS OF COAGULATIVE-REACTIVE AGENTS IN CANCER

The International Committee on Thrombosis and Hemostasis has established a Subcommittee on Hemostasis and Malignancy. This Subcommittee maintains a registry of therapeutic trials in which anticoagulants, platelet antagonists, inhibitors of prostaglandin synthesis, or modifiers of fibrinolytic reactions are being tested in human malignancy.

Information on such studies may be sent to Leo R. Zacharski, MD, VA Hospital, White River Junction, Vermont 05001.

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(Continued on following page)

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PREPARING THE MANUSCRIPT

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1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

Journal article, in press.

2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

Complete book:

3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

Chapter of book:

4. Moore G, Minowada J: Human hemopoietic cell lines A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4 Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed)· Gamma Globulins, Proceedings of the Third Nobel Symposium. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

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8 Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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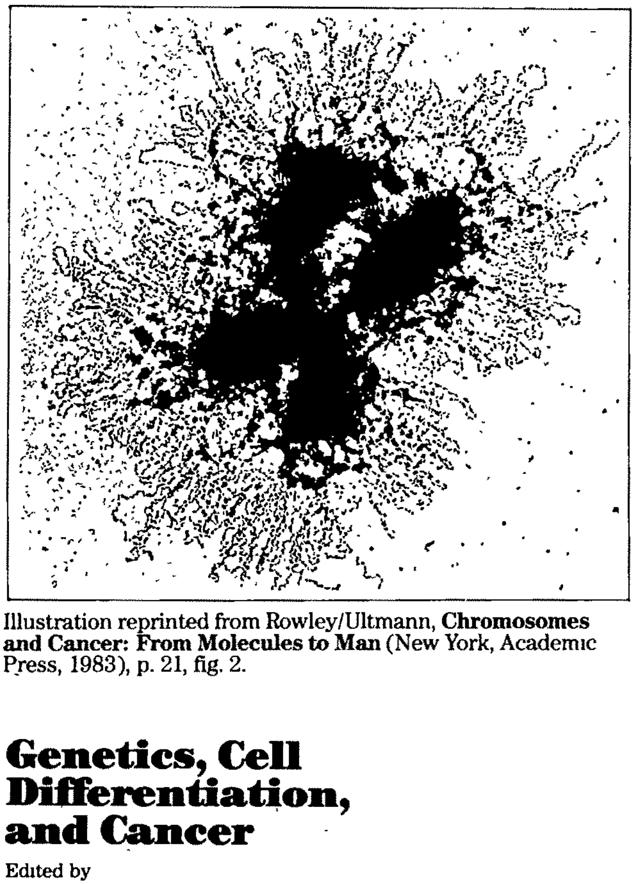

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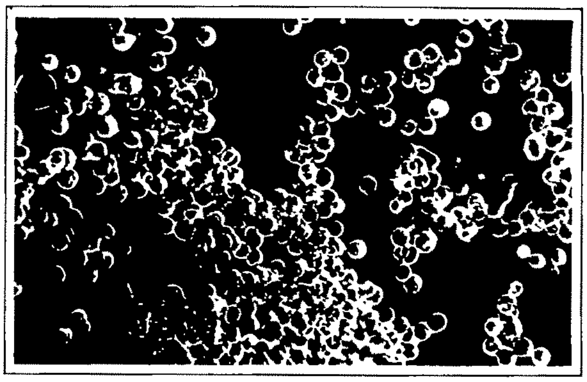

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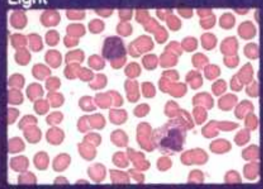
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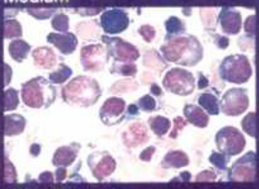
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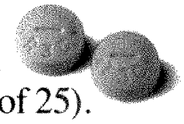

Consider Enhanced Quality of Life:

Often discharged from the hospital earlier, many patients can complete their course of therapy on an outpatient basis and return to a more normal, comfortable lifestyle sooner.

Consider Less Costly Care:

Mg for mg, Wellcovorin Tablets are less expensive than injectable leucovorin. Plus, with Wellcovorin Tablets, hospital stays are often shorter and clinic visits less frequent.

Consider Greater Patient Comfort:

Patients no longer need to drink special liquid formulations or endure a series of painful needle punctures. Patients simply take the prescribed number of Wellcovorin Tablets with water.

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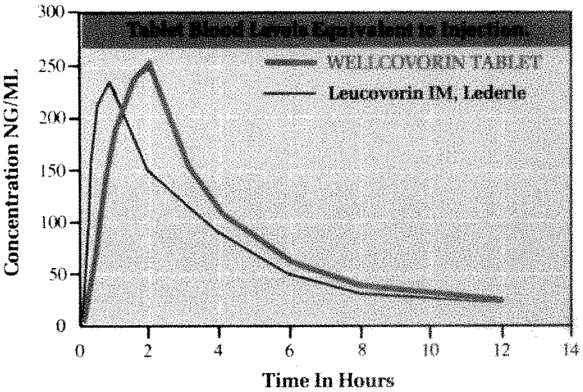
Consider Assured Blood Levels:

Wellcovorin Tablets are bioequivalent to equal amounts of injectable leucovorin, whether it is administered intravenously or intramuscularly (peak serum concentrations are reached after approximately two hours).

Blum MR, Guaspari A, Liao SHT, et al. Bioavailability and pharmacokinetics of oral and parenteral leucovorin. 33rd National Meeting Pharm Sci, San Diego, California, 1982, 12(2):175



Average serum folates after single 15 mg dose of leucovorin administered either IM or orally. Adapted from Blum, et al

Please see brief summary of prescribing information on next page.

WELLCOVORIN® TABLETS
(leucovorin calcium)
Leucovorin in convenient
5 mg and 25 mg tablets

Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer, 41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al*: High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med, 68*:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro*. *Br J Haematol, 40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al*: The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis. 135*(3): 493-496, 1977.

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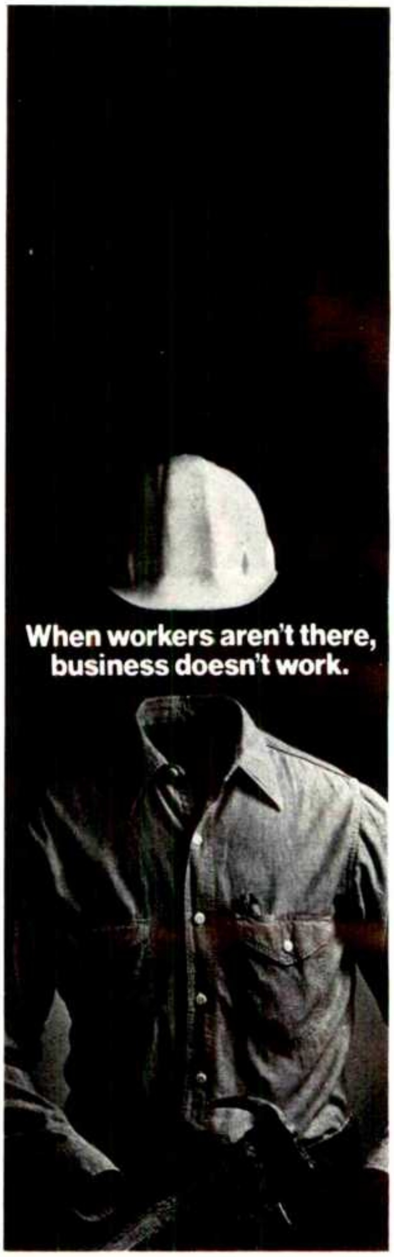

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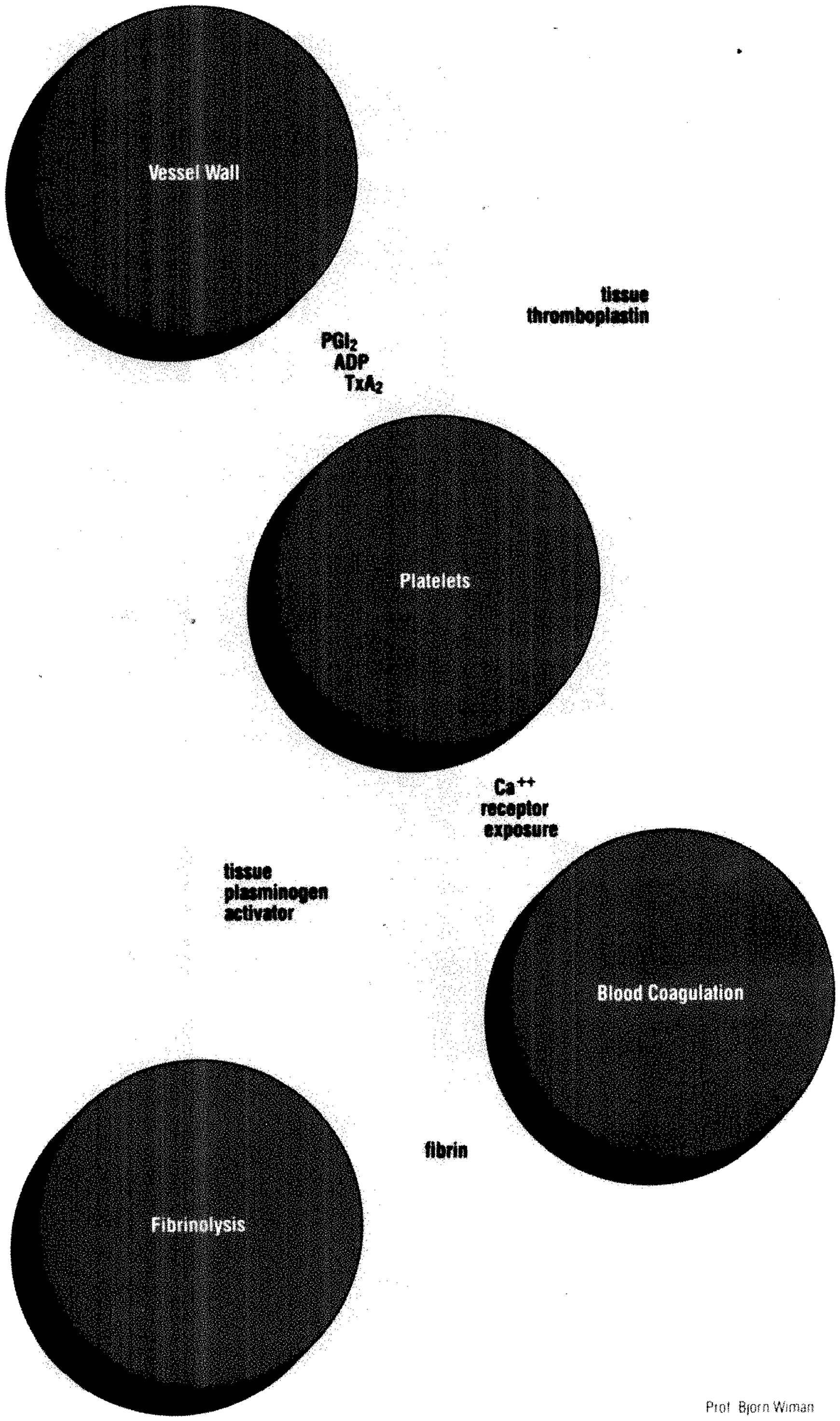

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MACOP-B

Drug Doses and Treatment Schedule for MACOP-B†

Drug	Dose	Route	Timing
Methotrexate††	400 mg/m²	IV	Weeks 2, 6, 10
Doxorubicin	50 mg/m²	IV	Weeks 1, 3, 5, 7, 9, 11
Cyclophosphamide	350 mg/m²	IV	Weeks 1, 3, 5, 7, 9, 11
Vincristine	1.4 mg/m²	IV	Weeks 2, 4, 6, 8, 10, 12
Bleomycin	10 U/m²	IV	Weeks 4, 8, 12
Prednisone	75 mg	Oral	Daily, dose tapered over the last 15 days
Co-trimoxazole	2 tablets	Oral	Twice daily throughout

† MACOP-B = methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin; IV = intravenous.

†† Methotrexate given as a 100 mg/m² intravenous bolus, then 300 mg/m² intravenously over 4 hours, followed 24 hours later by folinic acid, 15 mg orally, every 6 hours for 6 doses.

* Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med.* 102:596-602, 1985.

Blenoxane*

(sterile bleomycin sulfate)

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane* be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the post-operative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban.* It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

SUPPLY: Each vial contains 15 units of Blenoxane as sterile bleomycin sulfate. NDC 0015-3010-20

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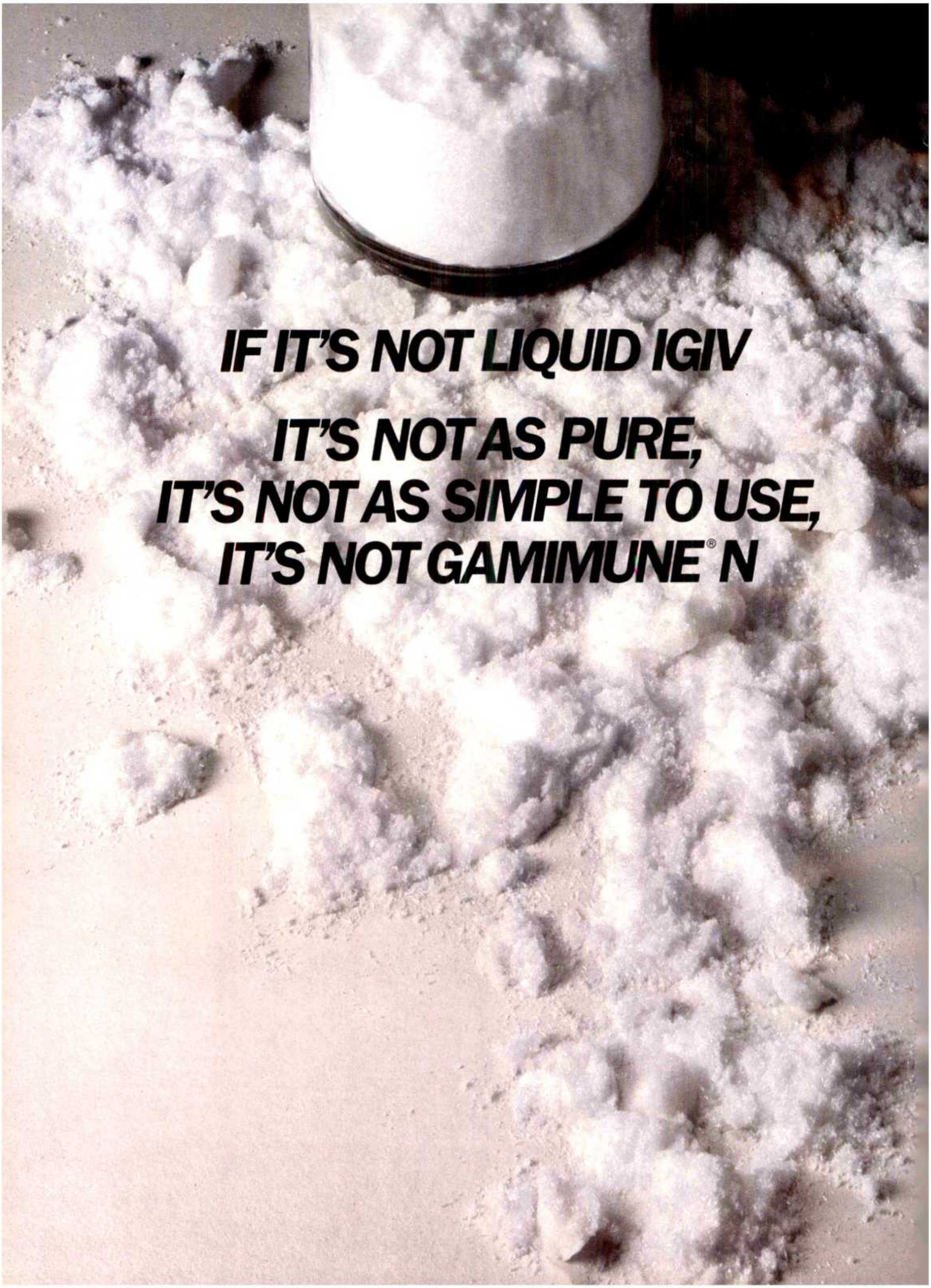
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*Established by studies in conjunction with the Centers for Disease Control. Data on file, Cutter Laboratories.

Please see following page for brief summary of prescribing information. 86 GAM 1

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GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 40% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION) is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25 – Gamimune® N is 16.5 mEq/L (~0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti-PL^{A1} specificity) also responded to treatment with Gamimune N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25 – Gamimune® N should be considered. In patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune N. It is not known whether Gamimune N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune N. Symptoms reported with Gamimune N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25 – Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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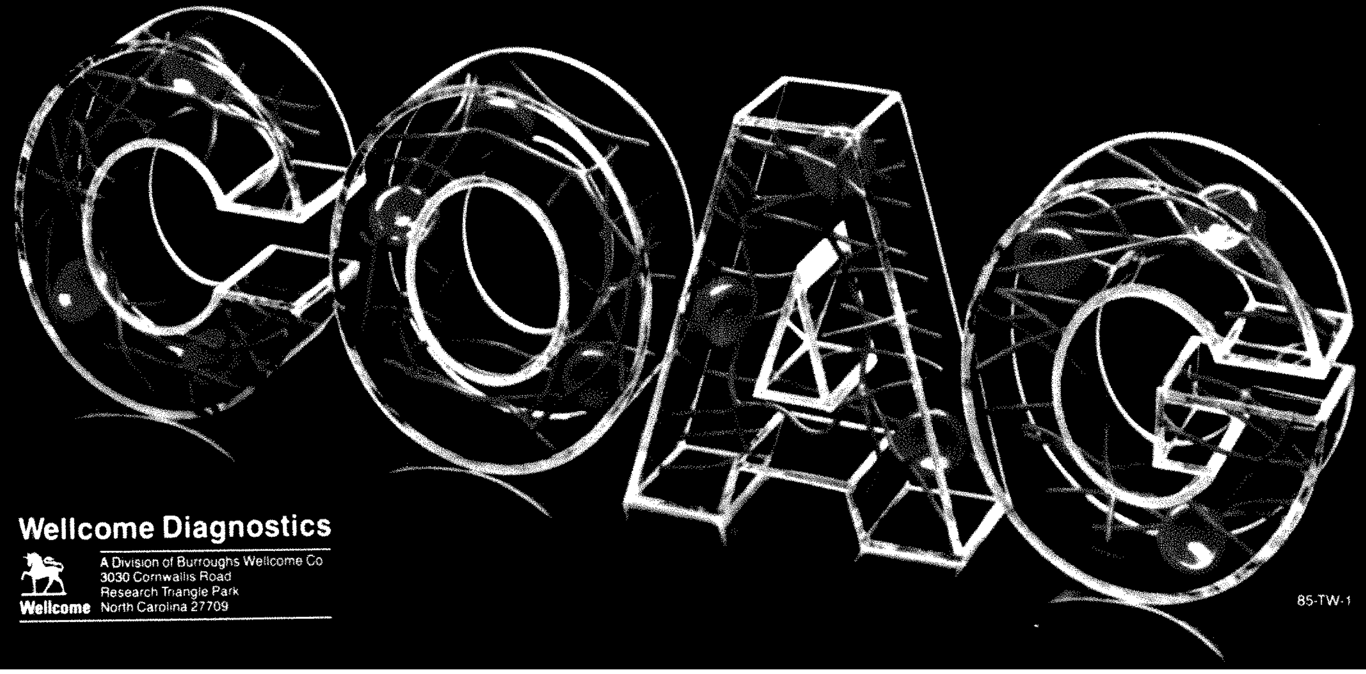

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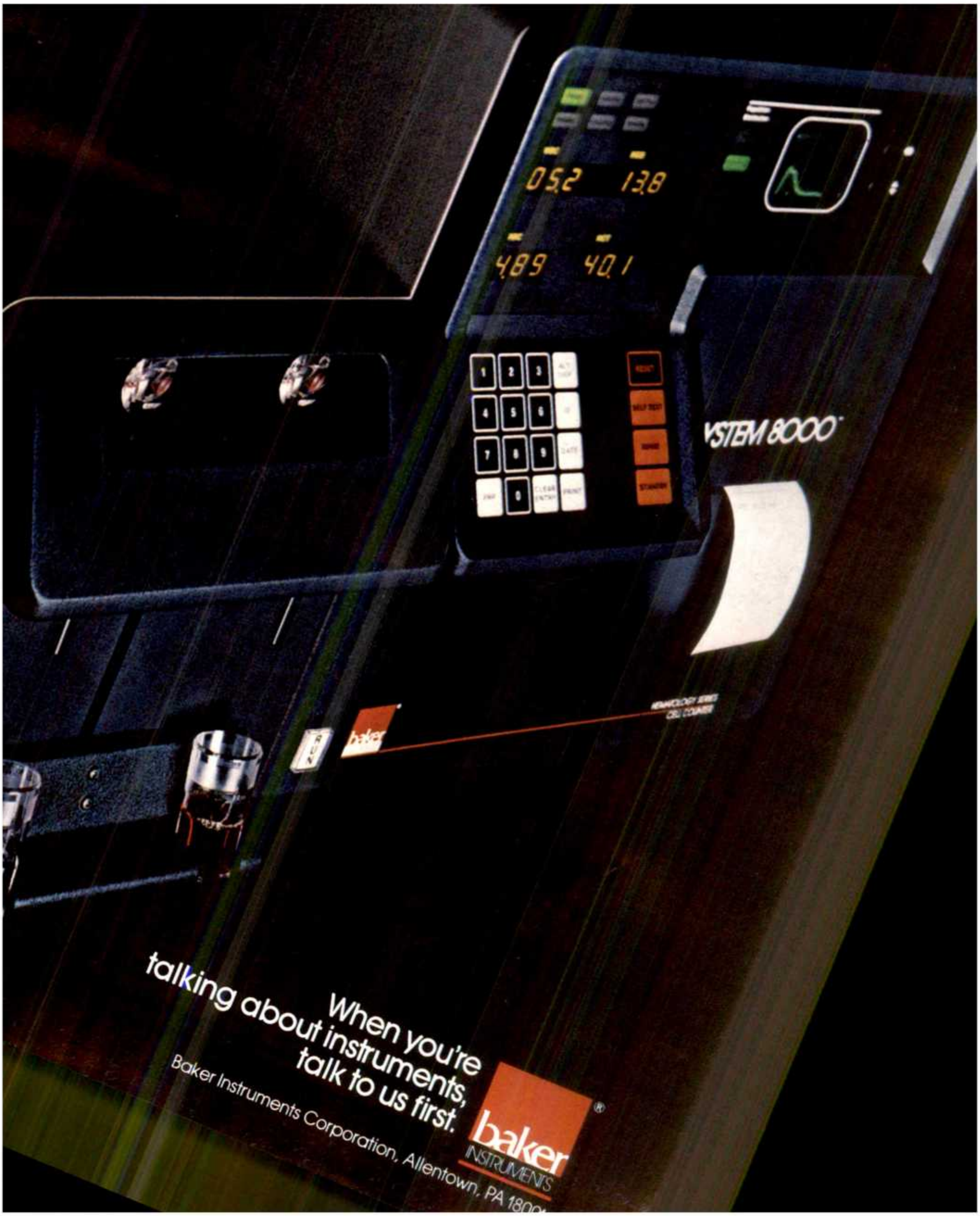
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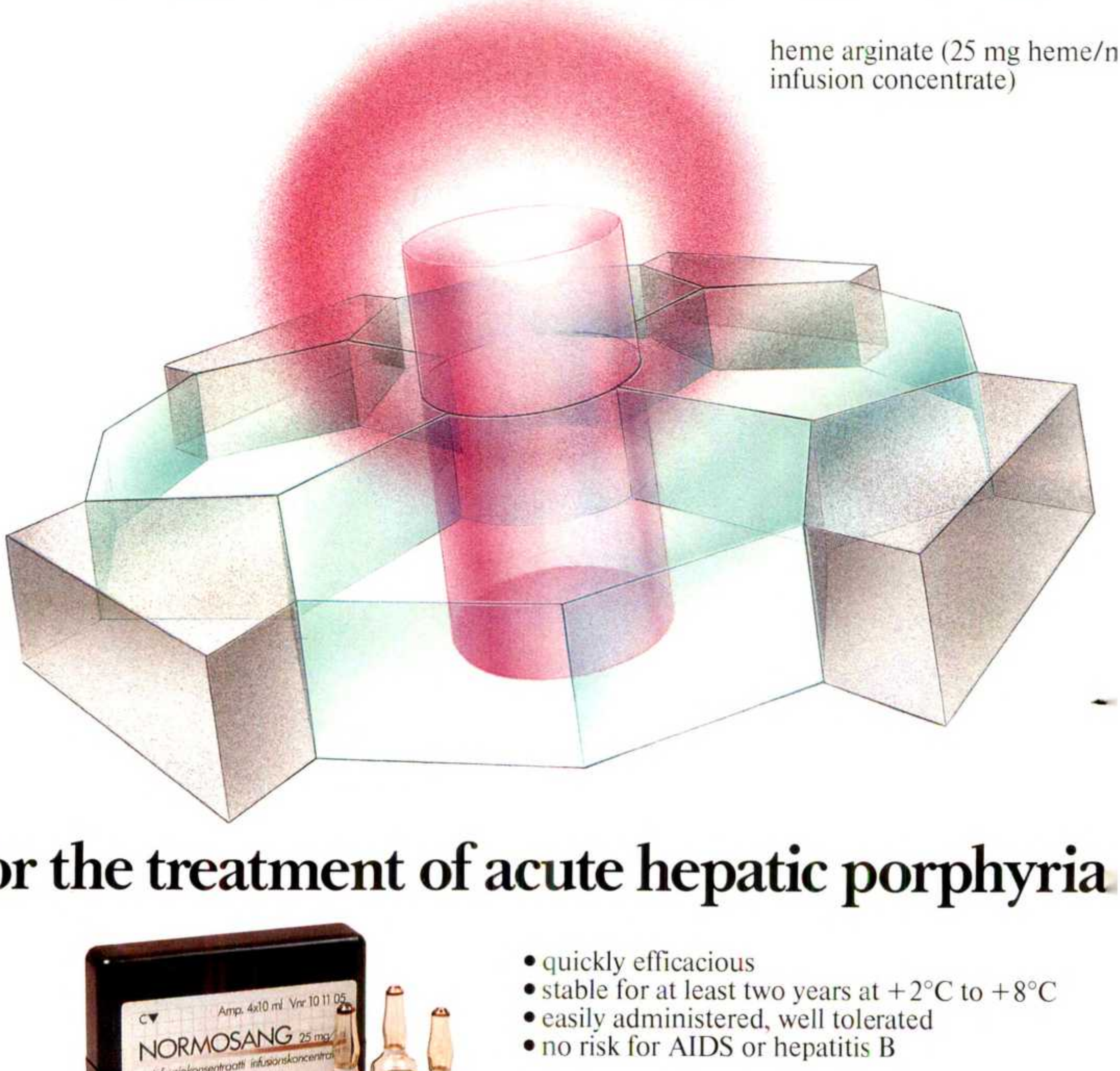

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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

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8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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WARNINGS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to lack of Vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

In the treatment of overdosage of folic acid antagonists, Leucovorin must be administered within one hour, if possible, and is usually ineffective if administered after a delay of four hours.

PRECAUTIONS: In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive.

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

DOSAGE: Megaloblastic anemia: No more than or up to 1 mg daily. There is no evidence that intramuscular doses greater than 1 mg daily have greater efficacy than those of 1 mg; additionally loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg.

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WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (eg, methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS: *General:* Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia, a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin, and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with leucovorin. It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Leucovorin should be given to a pregnant woman only if clearly needed.

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Pediatric Use: (See Drug Interactions.)

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for 72 hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the premethotrexate serum creatinine, the leucovorin dosage should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[7,8] The recommended dosage of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (ie, trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[9,10,11]

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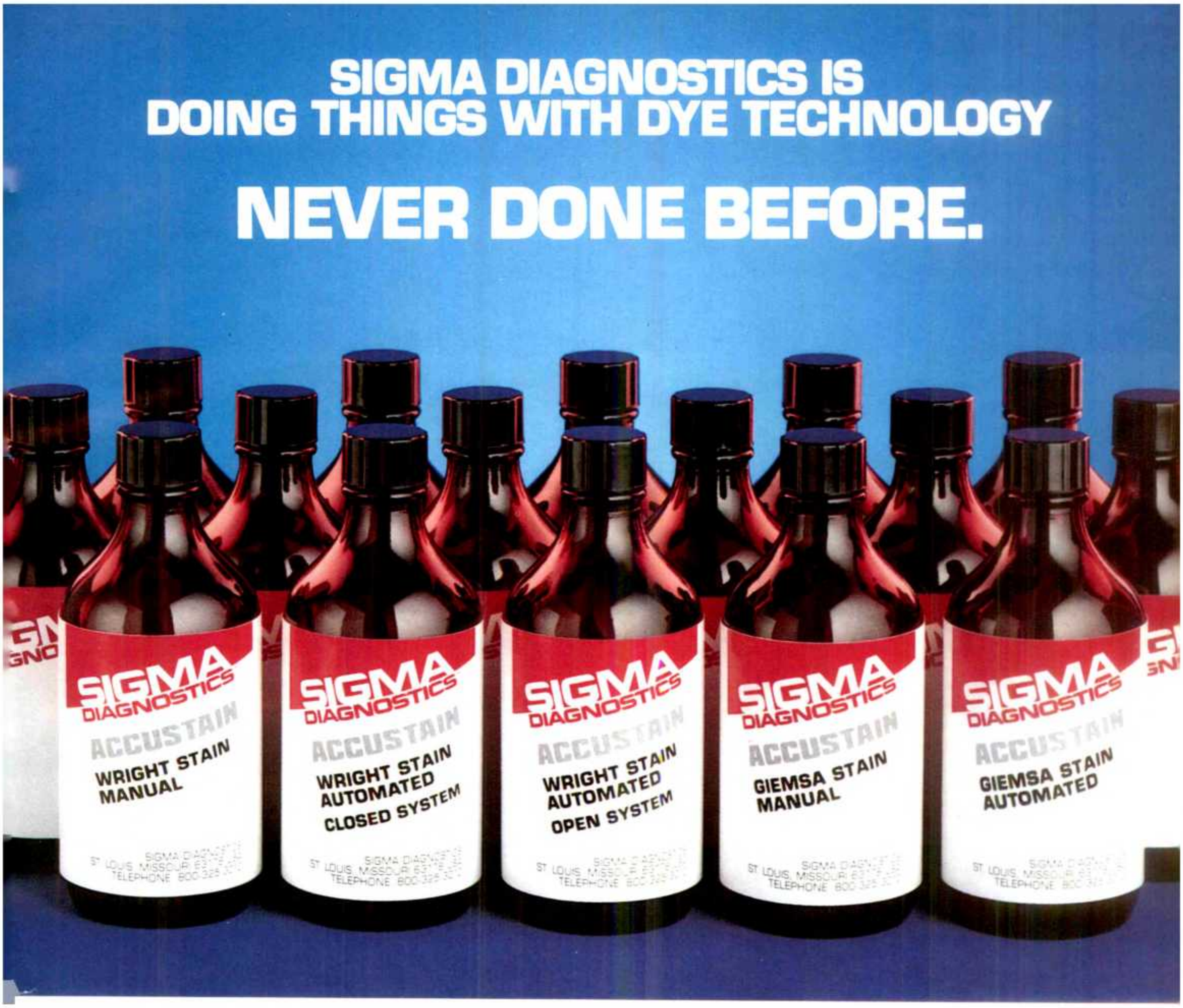

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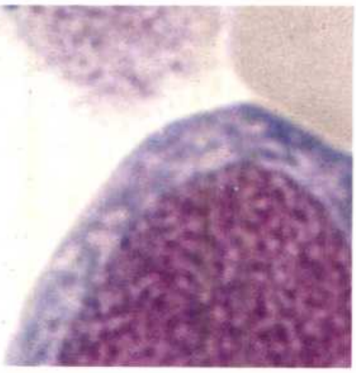

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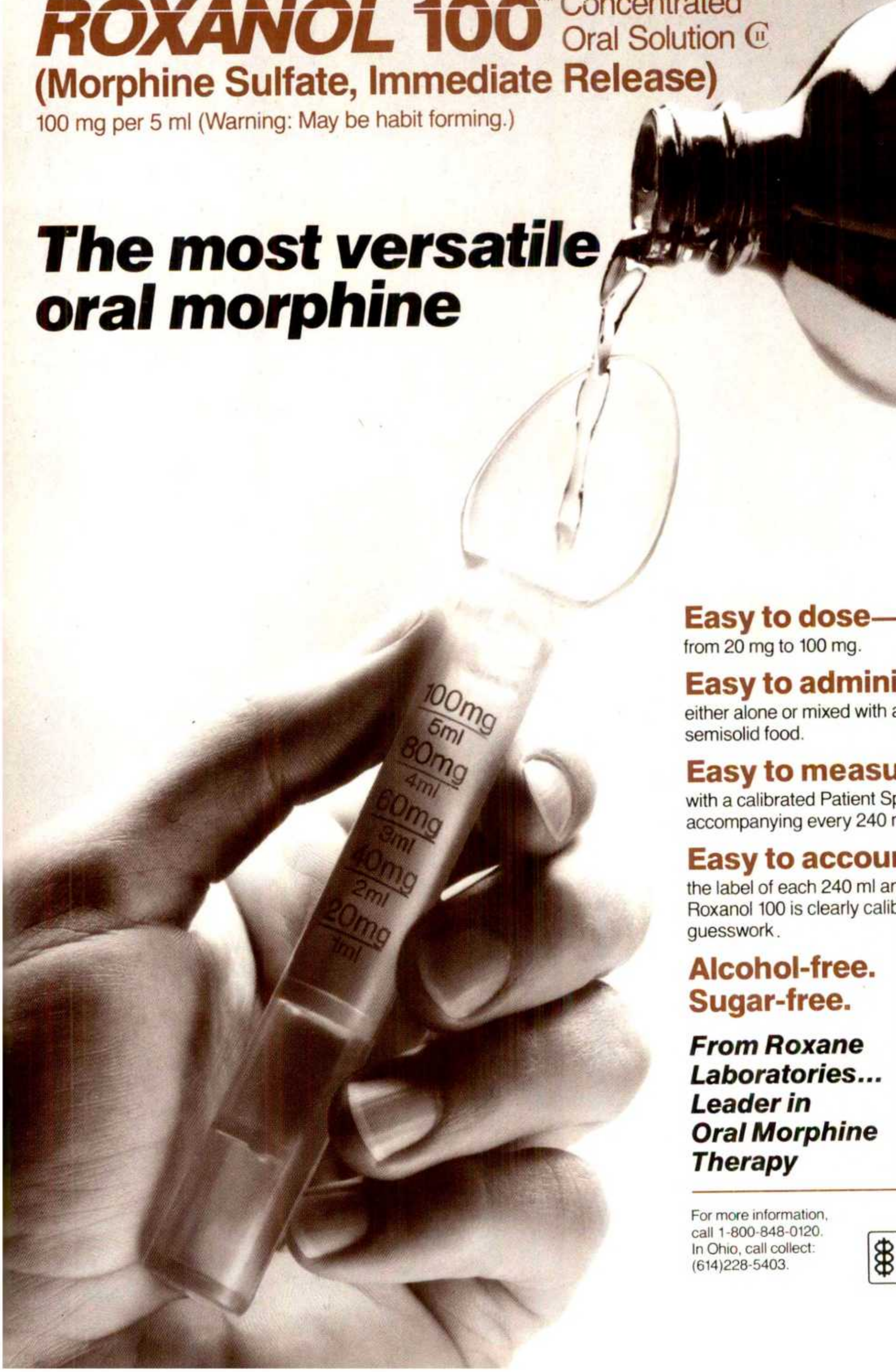

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REVIEW

Chromosomal Fragile Sites and Cancer-Specific Rearrangements

By Michelle M. Le Beau

IT IS NOW WELL established that human tumors, particularly leukemias and lymphomas, are characterized by nonrandom chromosomal abnormalities. During the past 2 years, a number of proto-oncogenes and transforming genes have been mapped to the chromosomal breakpoints involved in the specific structural rearrangements. Experimental evidence obtained recently suggests that the genes located at these breakpoints play an integral role in tumorigenesis. Another factor that has added to the interest in cancer-specific chromosomal abnormalities is the recent recognition that the breakpoints noted in the recurring structural rearrangements in human tumors have a remarkable concordance with the location of both heritable and common fragile sites. Moreover, several individuals with malignant diseases characterized by specific abnormalities have been identified as carriers of a fragile site at the point where a chromosome break occurred. Thus, the potential importance of fragile sites relative to genetic predisposition to the development of human tumors is substantial. For this reason, hematologists and oncologists should be aware of research efforts to clarify the role of fragile sites in the pathogenesis of human tumors. Scientific interest in this association is very high, although there are many unresolved issues.

In this review, I will examine the nature and characteristics of the chromosomal fragile sites with an emphasis on those sites that are heritable, the current theories regarding the mechanism(s) of fragile site formation, the correlation of fragile site location with the chromosomal breakpoints in the nonrandom abnormalities noted in human tumors, and the gaps in our current understanding of the role of fragile sites in the pathogenesis of human tumors.

HISTORICAL ASPECTS

The first report of a fragile site was published in 1965 by Dekaban who described a persisting clone of cells with an abnormal C group chromosome in a woman who had received radiation therapy.[1] Three years later, Lejeune et al demonstrated that these sites were heritable when they described a fragile site on the long arm of chromosome 2 in a woman and her daughter.[2] The chromosomal fragile site at Xq27, now known as the fragile X, was first described by Lubs in 1969 in association with mental retardation.[3] In the following years, it became clear that the fragile X chromosome is associated with, if not the cause of, one form of X-linked mental retardation in both males and females.[4]

The heritable chromosomal fragile sites were defined by Sutherland[5,6] as specific points or loci that show the following features: (1) the chromosome has a nonstaining gap of variable width that usually involves both chromatids; (2) the gap involves the same locus in any patient or kindred and is inherited in a Mendelian codominant fashion; (3) the fragility is expressed by the production of acentric fragments, deleted chromosomes, or triradial figures. It has been demonstrated with chromosome polymorphisms that the chromosome expressing the fragile site is always the same homologue. More recently, a second class of fragile sites has been recognized, namely, the common or constitutive fragile sites. The characteristics of this group of fragile sites are described in a later section.

In cytogenetic nomenclature, fragile sites are designated by the triplet "fra" followed by the chromosome number and the chromosome arm and band. For example, the fragile X chromosome may be identified as fra(X)(q27) where q is the long arm and 27 is the band number; p identifies the short arm of the chromosome. The morphologic appearance of one autosomal fragile site, fra(16)(q22), is illustrated in Fig 1.

In the past 10 years, a number of fragile sites have been identified; the key to the detection of these sites was the discovery that fragility may be expressed only under highly specific culture conditions and, in some cases, only in certain tissues. To elicit the expression of fragile sites, Sutherland[7] showed that it was necessary to culture lymphocytes in medium 199 rather than in a variety of other culture media that he examined. The essential feature of medium 199 was later found to be its deficiency in folic acid and thymidine.[5] Since Sutherland's observations, a number of compounds have been identified that can affect the expression of folic acid–sensitive fragile sites. It is well established that the

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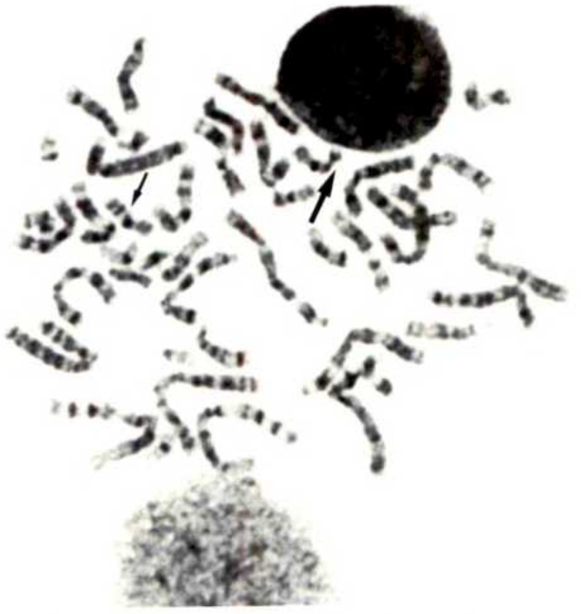

Fig 1. Trypsin-Giemsa banded metaphase cell illustrating the fra(16)(q22) (large arrowhead). A chromosome gap has occurred at band q22 of chromosome 16; the homologous no. 16 is normal (small arrowhead). Phytohemagglutinin-stimulated peripheral blood lymphocytes were cultured in medium 199 (folic acid–free) for 72 hours at 37 °C in a 5% CO_2/95% air atmosphere. Distamycin A (100 μg/mL, Sigma Chemical Co, St Louis) was added for the final 24 hours of incubation; metaphase cells were prepared using standard techniques.

antifolates methotrexate and aminopterin induce this expression,[5] as do the thymidylate synthetase inhibitors fluorodeoxyuridine (FdU), fluorodeoxycytidine (FdC), and trifluorothymidine.[8-10] Conversely, addition of folic acid, folinic acid, thymidine, and bromodeoxyuridine (BrDU) to the medium inhibits expression of the fragile X and folate-sensitive fragile sites in the autosomes.[5]

Several fragile sites are insensitive to the concentration of folic acid in the culture medium; however, each of these can be induced by the addition of certain compounds to the medium. For example, the fra(10)(q25) is expressed only if BrDU is present in the culture medium for at least the final eight to 24 hours.[11,12] The fra(16)(q22) and fra(17)(p12) may be expressed spontaneously in some individuals but are also induced by distamycin A, netropsin, BrDU, bromodeoxycytidine (BrDC), Hoechst 33258, interferon, and caffeine.[13-19]

In addition to the deficiency of folic acid or thymidine or to the presence of other compounds in the culture medium, a number of other factors affecting the expression of fragile sites have been identified. These include the pH of the medium, the duration of the culture, the serum concentration, the presence of methionine, the storage of cells prior to culture initiation, the harvesting technique used, and the method of slide preparation.[4] Because of individual variation and the effects of culture conditions, fragile sites are usually seen in less than 50% of metaphase cells prepared from phytohemagglutinin-stimulated (PHA) peripheral blood lymphocytes. Temporal variations in the percentage of cells expressing heritable fragile sites are commonly observed.

Fragile sites have been studied principally in PHA-stimulated lymphocytes; however, they have also been examined in a number of other tissues. Of the hematopoietic cells, some studies have shown that fragile sites can be expressed in bone marrow cells and in lymphoblastoid cell lines. Other investigations have demonstrated that skin fibroblasts and amniotic fluid cells may be used for the study of fragile sites, although the results have been variable.[19] Finally, expression of the fragile X has also been noted in somatic cell hybrids.[20]

CLASSIFICATION OF FRAGILE SITES

At present, there are 17 recognized heritable fragile sites; 16 of these are autosomal and the 17th is on the X chromosome. The heritable fragile sites can be grouped in three classes based upon the culture conditions necessary for their expression (Table 1).[21,22] Twenty-eight additional fragile sites have been recognized (23 common and five unclassified or possible rare fragile sites), bringing the total number to 45. The first group, that of folate-sensitive fragile sites, comprises 14 of the 17 recognized heritable fragile sites. As described previously, these sites are expressed under tissue culture conditions in which there is a relative deficiency of thymidine. This condition can be achieved by induction with the antifolates methotrexate or aminopterin and by specific inhibition of thymidine synthetase with FdU or FdC.[5,8-10] The folate sensitive fragile sites are 2q11, 2q13, 6p23, 7p11, 8q22, 9p21, 9q32, 10q23, 11q13, 11q23, 12q13, 16p12, 20p11, and Xq27.

The second group, the distamycin A–inducible fragile sites, includes the fra(16)(q22) and the fra(17)(p12). These fragile sites may be expressed spontaneously; however, their expression is enhanced by the presence of distamycin A, interferon, BrDU, netropsin, or Hoechst 33258 in the culture medium.[13-19] The sole member of the third group is the BrDU-requiring fragile site at 10q25, which is expressed only if BrDU is present in the culture medium for at least the final eight to 24 hours of culture.[11,12] The fra(10)(q25) is not induced by the other compounds that induce the group 2 fragile sites.

It is difficult to estimate the frequency of the heritable fragile sites (groups 1 to 3) because in most cytogenetic surveys of newborns and other populations the medium used was not optimal for the expression of fragile sites. However, these fragile sites are generally very rare. The most common is the BrDU-induced site at 10q25, which is present in 2 to 3% of the Australian population.[22,23] Thus, this fragile site can be regarded as a human polymorphism. Estimated frequencies of other fragile sites are 1/200 to 1/700 for the folate-sensitive fragile sites and 1/100 for the fra(16)(q22).[22]

Recently, a fourth group of fragile sites has been described, the common or constitutive fragile sites (Table 1). Our understanding of the nature of these fragile sites is limited; however, they appear to occur in humans with a very high frequency. The common fragile sites may be induced by low folate levels, but expression is enhanced by chemicals such as aphidicolin and caffeine. At the Seventh International Workshop on Human Gene Mapping,[21] three chromosomal regions were classified as common fragile sites,

Table 1. Fragile Sites on Human Chromosomes

Heritable fragile sites, groups 1–3	
Group 1, folate-sensitive fragile sites	2q11
	2q13
	6p23
	7p11
	8q22
	9p21
	9q32
	10q23
	11q13
	11q23
	12q13
	16p12
	20p11
	Xq27
Group 2, distamycin A–inducible fragile sites*	16q22
	17p12
Group 3, BrDU-requiring sites	10q25
Common fragile sites, group 4	1p36
	1p32
	1p22
	1q25
	2p24
	2p13
	2q31
	2q33
	3p14
	3p24
	3q27
	5q31
	6q21
	6q26
	7p13
	7q22
	7q32
	11p13
	14q24
	16q23
	22q12
	Xp22
	Xq22
Unclassified or possible rare fragile sites, group 5	1q32
	2p11
	5q35
	Xq26
	Yq12

Includes those fragile sites that were accepted at the Seventh International Workshop on Chromosomes in Leukemia and those listed as common fragile sites in ref 22.

*Expression of group 2 fragile sites is also induced by BrDU, netropsin, Hoechst 33285, and interferon

namely, 3p14, 6q26, and 16q23.[24–28] However, the actual number of common fragile sites may be much higher, as suggested in recent studies by Glover et al[29] who observed breaks at 25 sites induced by aphidicolin, by Yunis and Soreng[30] who described fragility at 51 sites in cells deprived of thymidine and exposed to caffeine, and by Barbi et al[31] who noted 11 folic acid–sensitive sites in cells exposed to methotrexate. The common fragile sites and the chemicals that enhance their expression are reviewed by Sutherland and Hecht.[22]

There are a number of important distinctions between the heritable and the common fragile sites. The heritable fragile sites are rare and segregate in a simple Mendelian fashion with complete penetrance; the common fragile sites, on the other hand, are frequent, if not ubiquitous, and may be caused by environmental factors such as chemicals, radiation, and/or viruses. Moreover, the heritable fragile sites are consistently present in one homologue and are usually manifested as chromosome-type aberrations with chromosome breaks, deletions, and triradials. The common sites may be observed in both homologues, often in the same metaphase cell, and they predominantly consist of chromatid-type lesions. Whether there are additional compounds that enhance the expression of other common sites remains to be determined. Fragility at sites also known to be heritable fragile sites, eg, 8q22 and 16q22, was observed in cells exposed to caffeine or aphidicolin.[29,30] It is unknown whether the heritable fragile sites and the lesions induced by these compounds are identical at the DNA level.

MECHANISMS OF FRAGILE SITE FORMATION

At present, the precise mechanism(s) of fragile site formation is unknown. Initially, it was suggested that these lesions arose simply from despiralization of chromosomes that is mediated by alterations in DNA, histones, nonhistone proteins, or divalent cations.[32] The nature of the inhibitors and inducers of the folate-sensitive fragile sites suggests, however, that the process is likely to operate during DNA synthesis; the common feature of these agents is that they interfere with the 5,10-methylenetetrahydrofolate-requiring conversion of deoxyuridylate to thymidylate. Sutherland[5] proposed that the fragile site is created because a section of thymidine-rich DNA cannot complete synthesis when the thymidine supply is restricted and that the site subsequently does not compact normally during mitosis. It now appears that the actual mechanism may be more complex.

Goulian et al[33] have demonstrated that when the level of thymidine in the medium is low, uracil is misincorporated into DNA during replication. This finding led investigators to propose that the folate-sensitive fragile sites appear as a result of heritable defects of DNA methylation along a chromosome region that normally binds a folding protein involved in chromosome condensation.[34] The misincorporation of uracil in place of thymine into DNA would produce a further loss of methyl groups, eventually precluding a full DNA-folding protein interaction; the result would be a localized collapse of the chromosome structure.

An alternative interpretation of the effects of uracil incorporation into DNA on fragile site formation has also been described.[35,36] The mechanism is based upon the phenomenon of thymidine less death observed in bacteria.[35] The incorporated uracil is subject to recognition and removal by uracil-*N*-glycosylase, and the resulting apyrimidinic sites would be attacked by endonucleases. Each strand break would serve as a site for initiation of an excision-repair patch; in the absence of thymidine, dUMP would be incorporated and removed cyclically, with lengthening of the patches during successive cycles. Ultimately, double-strand breakage would result in fragmentation of the chromosome. Whether the folate-sensitive fragile sites are the consequence of an abortive repair process or of the presence of an altered base remains to be determined.

Additional hypotheses for the formation of fragile sites include that of viral modification of DNA[37] as well as the theory of Lejeune (personal communication, September 1984) that the relative decondensation of chromatin is related to a high rate of transcriptional activity of genes that are specifically induced by folate deprivation. This transcriptional activity would be maintained during early prophase, thereby preventing chromosome condensation. With regard to the former hypothesis that fragile sites represent sites of viral modification of DNA, it is known that infection of permissive cells with adenovirus 12 results in decondensation of three consistent chromosome regions (1p36, 1q21, 17q21). Moreover, Lindgren et al recently mapped to these chromosome regions a multigene family coding for the small nuclear RNA molecules U1 and U2, which have been implicated in RNA processing.[38] They have suggested that the small nuclear RNA genes are the major targets of viral chromosome modification. It is still unknown whether other chromosomal regions such as the fragile sites may also be targets of viral modification.

The relationship of the aforementioned hypotheses to the mechanisms causing the fragile sites inducible by distamycin A and BrDU and to those causing the common fragile sites is unclear. Distamycin A, Hoechst 33258, and netropsin preferentially bind to A-T clusters in DNA and may lead to differential inhibition of chromosome condensation.[39] It may be that BrDU is incorporated into DNA and, like the other three agents, causes conformational changes in the DNA. Moreover, the theory of Lejeune may not be restricted to the folate-sensitive fragile sites in that conformational changes in DNA caused by agents such as distamycin A may also cause alterations in transcriptional activity.

At present, the mechanism by which interferon induces expression of the fra(16)(q22) has not been determined. With regard to the possibility that fragile sites are regions of DNA with a high rate of transcriptional activity, it is of interest that α- and β-interferons induce transcription of the functional metallothionein genes. As described in a later section, the metallothionein genes have been mapped to 16q22; the breakpoint at q22 in the inv(16) and t(16;16) associated with acute myelomonocytic leukemia occurs within the cluster of metallothionein genes, and some of these patients have been found to be carriers of the fragile site at 16q22.

Regarding the common fragile sites, caffeine is believed to induce the synthesis of proteins required for mitosis during the G2 phase, causing cells to enter mitosis before proper repair of lesions in their DNA.[40,41] In experiments on induction of fragile sites by caffeine, cells have been cultured in folate-depleted medium or in the presence of FdU, suggesting that incorporation of uridine into DNA and the subsequent effects of this incorporation may play a role. Aphidicolin, the second chemical known to enhance the expression of the common fragile sites, is an inhibitor of DNA polymerase α and thus may alter the synthesis of DNA in specific chromosome regions.

BIOLOGIC SIGNIFICANCE OF FRAGILE SITES

The association of fragile sites with phenotypic abnormalities is well established for the fra(X), which is associated with one form of X-linked mental retardation that is characterized by macroorchidism and connective tissue dysplasia in affected males. The phenotypic consequences of the autosomal fragile sites are less clear. In general, they are considered to be normal chromosomal variants despite sporadic reports of the presence of fragile sites in individuals who have congenital abnormalities. Recently, fragile sites have been implicated in the pathogenesis of human tumors. In 1983, Yunis found a correlation between three fragile sites and chromosomal rearrangements in malignant cells.[42] Four of five cancer patients studied were found to be carriers of a fragile site in a chromosome region that was also involved in a rearrangement in their malignant cells. Yunis proposed that chromosomal rearrangements might be facilitated by the presence of heritable or constitutional fragile sites.

Le Beau and Rowley[43] identified a remarkable concordance between the chromosomal location of heritable fragile sites and the breakpoints that occur in the nonrandom abnormalities associated with leukemia and lymphoma. This observation was subsequently noted by other investigators.[44,45] Moreover, Hecht and Sutherland[46] determined that there was a statistically significant association between the 21 fragile sites and 50 cancer-specific breakpoints accepted by the Seventh Human Gene Mapping Workshop.[21]

Figure 2 summarizes the location of the 21 fragile sites accepted by the Seventh Human Gene Mapping Workshop as well as the proto-oncogenes and transforming sequences

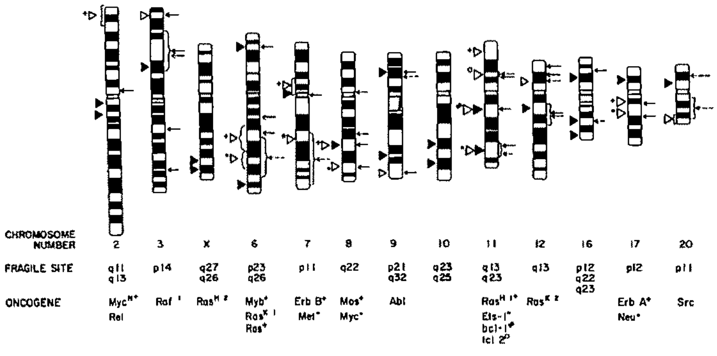

Fig 2. Diagram of chromosomes containing known fragile sites. The chromosome number, fragile site, and proto-oncogene or transforming sequence that has been mapped to these chromosomes are indicated below each chromosome. The arrowheads to the left of each chromosome indicate the bands carrying the fragile site (▶) or proto-oncogene (▷); the arrows to the right of the chromosomes identify specific bands involved in consistent translocations (←) or deletions (←---) that are observed in human tumors. Additional symbols (+, •, #, O) identify the proto-oncogenes or transforming sequence and their location.

that have been mapped to these chromosomes. The nonrandom changes affecting the chromosomes and the malignant diseases in which the karyotypic abnormalities are frequently seen are listed in Table 2. Twenty-four of the nonrandom chromosomal abnormalities observed in leukemia and lymphoma involve eleven regions known to contain heritable fragile sites in some individuals. One additional common fragile site has been localized to a region that is involved in chromosomal rearrangements in solid tumors.

The nonrandom abnormalities in leukemia and lymphoma in which breakpoints coincide with fragile sites are summarized in the following paragraphs. Band p23 of chromosome 6 contains a fragile site and is also the breakpoint in the t(6;9)(p23;q34) which is associated with bone marrow basophilia in ANLL.[47,48] A new recurring translocation, t(1;7)(p11;p11), noted in patients with myeloid disorders (ANLL, MPD, or a MDS) involves a breakpoint at 7p11, which is known to be a fragile site.[49] The t(8;21)(q22;q22)

Table 2. Chromosomal Aberrations Involving Chromosomes Containing Fragile Sites

Chromosome*	Rearrangement	Disease	Fragile Site
2	t(2;8)(p11,q24)	BL, ALL-L3	—
	t(2;14)(p11,q32)	NHL, CLL	—
	t(2;11)(p21;q23)	MDS, ANLL	11q23
	t(2;13)(q37;q14)	rhabdomyosarcoma	—
3	t(3;3)(q21;q26)	ANLL	—
	inv(3)(q21q26)	ANLL	—
	t(3;17)(q26;q22)	MPD	—
	t(1;3)(p36;q21)	MDS	—
	t(3;5)(q21-25;q31-35)	ANLL	—
	del(3)(p14p23)	SCLC, rhabdomyosarcoma, ovarian carcinoma, renal cell carcinoma	3p14
	t(3;11)(p13-14;p15)	renal carcinoma	3p14
	t(3;8)(p14;q24)	renal carcinoma	3p14
	t(3;8)(p21;q12)	parotid gland carcinoma	—
6	t(6;9)(p23;q34)	ANLL	6p23
	t(6;11)(q27;q23)	ANLL	11q23
	del(6q)	ALL, NHL, ATL, malignant melanoma	6q26
	t(6;14)(q21;q24)	cystadenocarcinoma of ovary	—
	t(6,12)(q15,p13)	PLL	—
7	del(7q)	ANLL, MDS	—
	t(1;7)(p11;p11)	MPD, MDS, ANLL	7p11
8	t(8;16)(p11;p13)	ANLL	—
	t(8;21)(q22;q22)	AML-M2	8q22
	t(8;9)(p11;q34)	MPD	—
	t(8;14)(q24;q32)	BL, ALL-L3	—
	t(2,8)(p11;q24)	BL, ALL-L3	—
	t(8;22)(q24;q11)	BL, ALL-L3	—
	t(8;14)(q22;q32)	NHL	8q22
	t(8;14)(q24;q11)	CLL, ALL, NHL	—
	t(8;10)(q12;p14)	lymphoproliferative disorders	—
	t(3;11)(p13-14;p15)	renal carcinoma	3p14
	t(3;8)(p14;q24)	renal carcinoma	—
	t(3,8)(p21;q12)	parotid gland carcinoma	—
	i(8q), del(8p)	bladder carcinoma	—
9	t(9;22)(q34;q11)	CML, ALL	—
	t(6,9)(p23;q34)	ANLL	6p23
	t(9;11)(p21;q23)	AMoL	9p21, 11q23
	t(8;9)(p11;q34)	MPD	—
	del(9p)	ALL	9p21
	del(9q)	ANLL, MDS, MPD	9q32
	t(9;21)(p24;q21)	ATL	—
10	ins(10;11)(p11;q23q24)	AMoL	11q23
	t(10;11)(p14,q13-14)	AMoL, AMMoL	11q13
	t(10,14)(q24-5;q11)	ALL, NHL	10q25
	t(8;10)(q12,p14)	lymphoproliferative disorders	—
	del(10)(q24)	prostate carcinoma	—

Table 2. Chromosomal Aberrations (Cont'd)

Chromosome*	Rearrangement	Disease	Fragile Site
11	del(11p)	Wilms' tumor	—
	t(11;14)(q13;q32)	ALL, NHL, CLL	11q13
	t(11;14)(p13;q11)	ALL, NHL	—
	t(11;14)(q23,q32)	ALL, NHL	11q23
	t(4;11)(q21;q23)	ALL	11q23
	t(10,11)(p14;q13-14)	AMMoL, AMoL	11q13
	t(11;17)(q23;q25)	AMMoL, AMoL	11q23
	t(9,11)(p21;q23)	AMoL	9p21, 11q23
	t(11;19)(q23,p13)	AMoL	11q23
	t(11;#)(q23,#)	AMoL	11q23
	del(11q)	AMoL	11q23
	t(6;11)(q27;q23)	ANLL	11q23
	t(2;11)(p21,q23)	MDS, ANLL	11q23
	t(11;22)(q23 or q24,q12)	Ewing's sarcoma	11q23
12	del(12p), t(12p)	ALL, ANLL	—
	t(6;12)(q15;p13)	PLL	—
	dup(12)(q13;q22)	NHL	12q13
	del(12q), t(12q)	parotid gland carcinoma	12q13
	i(12p)	seminoma, testicular carcinoma	—
16	inv(16)(p13q22)	AMMoL	16q22
	t(16;16)(p13;q22)	AMMoL	16q22
	del(16)(q22)	ANLL	16q22
	t(8,16)(p11;p13)	ANLL	—
17	t(15;17)(q22;q21)	APL	—
	i(17q)	CML, ANLL	—
	dic(17)(p12)	CML, ANLL	17p12
	t(1,17)(p11;q11)	CML	—
	t(3;17)(q26;q22)	MPD	—
	t(11;17)(q23;q25)	AMMoL, AMoL	11q23
	t(1;17)(p36;q21)	ANLL	—
	t(14;17)(q32,q23)	CLL	—
20	del(20)(q11q13)	ANLL, MPD, MDS	—
	del(20)(p12)	multiple endocrine neoplasia, type 2	—

Abbreviations: BL, Burkitt's lymphoma; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; ANLL, acute nonlymphocytic leukemia; MPD, myeloproliferative disorder; SCLC, small-cell lung carcinoma, ATL, adult T cell leukemia; PLL, prolymphocytic leukemia; AML, acute myeloblastic leukemia; CML, chronic myelogenous leukemia; AMOL, acute monoblastic leukemia; APL, acute promyelocytic leukemia; AMMoL, acute myelomonocytic leukemia.

*Includes only the 21 fragile sites that were accepted at the Seventh International Workshop on Human Gene Mapping[21]

observed in AML with maturation (AML-M2)[50] may be associated with a fragile site that has been identified at 8q22. A fragile site in no. 9 at p21 is also the breakpoint in the t(9;11)(p21 or p22;q23), which has been noted primarily in AMoL.[51] Translocations and deletions involving 9p21 have also been described in patients who had ALL with lymphomatous features.[52] Chromosome 10 contains two fragile sites (10q23,10q25); however, only one of these bands is the breakpoint in a cancer-specific rearrangement. This abnormality, the t(10;14)(q24-25;q11), has recently been observed in patients with ALL or NHL of T cell origin.[53]

There are two fragile sites on no. 11 (q13 and q23), each of which may be the breakpoint in various translocations or deletions in AML and ALL and in lymphoma. These include the t(11;14)(q13;q32) or t(11;14)(q23;q32) in NHL[54] as well as t(9;11)(p21;q23), t(11;19)(q23;p13orq13), ins(10;11)(p11;q23q24), del(11)(q23) and t(11q) in AMoL and AMMoL,[55,56] and t(4;11)(q21;q23) in ALL.[57,58] In no. 16, the fragile site at q22 occurs in the band that is affected in the inv(16)(p13q22), del(16)(q22), and t(16;16)(p13;q22) found in AMMoL characterized by bone marrow eosinophilia and abnormal eosinophil morphology and cytochemical reactions.[59-61] Another fragile site on no. 16, at p12, does not coincide with the short-arm breakpoint in the inv(16) and t(16;16) that has been identified as p13 in prophase chromosomes. Finally, an isodicentric chromosome resulting from a break in the short arm of chromosome 17 has been observed in patients with CML in the acute phase, and in patients with ANLL.[62]

One additional fragile site is located in a chromosomal region that may be rearranged in solid tumors; an interstitial deletion of 3p [del(3)(p14p23)] has been observed in some cell lines derived from patients with SCLC,[63] and translocations involving 3p14 have been noted in familial renal carcinoma [t(3;8)(p14;q24),t(3;11)(p13-14;p15)]. Two chromosome regions, 11q23 and 12p13, are involved in hematopoietic neoplasms as well as in solid tumors; the t(11;22)(q23orq24;q12) has been described in Ewing's sarcoma,[64,65] and translocations and deletions involving 12q13 have been noted in salivary gland tumors.[66]

Thus, 12 of the 21 recognized fragile sites (including 11 of the 17 heritable fragile sites) occur in chromosome bands that are involved in nonrandom abnormalities in human tumors. The association of cancer-specific breakpoints and fragile site locations has been analyzed statistically by Hecht and Sutherland.[46] By using a chi-square test, these authors determined that there was a highly significant statistical association ($P < .001$) between the location of the 21 fragile sites and the 50 recurring cancer breakpoints accepted by the Seventh Human Gene Mapping Workshop.[21]

If a relationship exists between the consistent chromosomal rearrangements in malignant cells and heritable fragile sites, one might expect that those patients whose malignant cells have one of these rearrangements would also be carriers of fragile sites that affect the involved chromosomes. Data from several sources suggest that this may in fact be the case. In 1983, Sessarego et al[67] described the presence of a fra(11)(q13) in peripheral blood lymphocytes of a patient who had myelofibrosis with myeloid metaplasia. An interstitial deletion of 11q [del(11)(q13q21)] with a breakpoint in the same (11q13) band was present in circulating immature cells.

Yunis[42,44] found a correlation between three fragile sites and chromosomal rearrangements in five cancer patients. Two patients with small-cell lymphoma had a t(11;14)(q13;q32) in their malignant cells; in one of these patients, analysis of PHA-stimulated lymphocytes revealed a fragile site at 11q13. A third lymphoma patient who had a t(12;14)(q13;q32) also had a fragile site at 12q13, and both patients studied who had AMMoL and an inversion of chromosome 16, inv(16)(p13q22), carried a fragile site at 16q22. In one of the latter patients, the presence of a heterochromatin variant enabled Yunis to determine that the homologue that was rearranged in the malignant cells also expressed the fragile site. In contrast, of three patients with AMMoL and an inv(16) (two patients) or del(16)(q22) (one patient) studied during complete remission by Arthur and Bloomfield,[68] only one patient was found to have a fragile site at 16q22.

Le Beau and Rowley[43] reported the presence of a fragile site at 16q22 in lymphocytes from four of five patients with AMMoL and an inv(16) who were studied while they were in remission. Chromosome breakage at 16q22 was not observed in any of the five healthy individuals analyzed as controls. We have expanded our studies to include 11 AMMoL patients whose malignant cells were characterized by an abnormality of no. 16 [inv(16), nine patients; del(16), one patient; t(16;16), one patient], and we found that ten of the 11 patients when examined during complete remission were carriers of a fragile site at 16q22. Figure 3 illustrates the inverted chromosome 16 observed in the malignant cells and the fragile site at 16q22 from normal lymphocytes of one of these patients. The parents of one patient with a fra(16) were also examined for the presence of fragile sites. We found that the father was a carrier of a distamycin A–inducible fragile site at 16q22, thereby indicating that, in at least this patient, the fragile site was a heritable site. Arthur et al[69] have also demonstrated the familial nature of the fragile site at 16q22 in a previously reported patient.[68] Specifically, these authors found that the mother and four children of a woman with AMMoL and an inv(16), who also had a fra(16), were also carriers of this fragile site. As mentioned earlier, the estimated frequency of the fra(16)(q22) in the general population is approximately 1/100 individuals; thus, it is unlikely that this fragile site would be observed randomly in ten of 11 patients who have a rearrangement involving 16q22 in their leukemic cells.

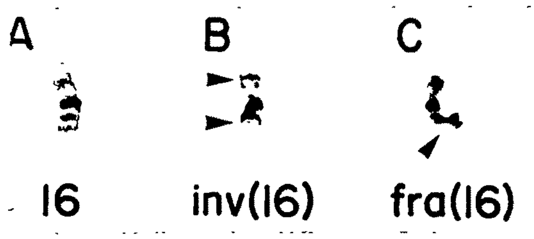

Fig 3. Partial karyotypes of trypsin-Giemsa banded metaphase cells illustrating the (A and B) normal and inverted [inv(16)(p13q22)] chromosome 16 homologues obtained from the malignant bone marrow cells of a patient with acute myelomonocytic leukemia. (C) The fragile 16 [fra(16)(q22)] observed in phytohemagglutinin-stimulated lymphocytes from this patient obtained at the time of complete remission. The chromosomal breakpoints of the inv(16) and the fragile site at 16q22 are identified with arrows.

In addition to the heritable fragile sites, the common fragile sites have recently been implicated as predisposing factors in the development of neoplasia. By culturing cells in the presence of aphidicolin or in folic acid–depleted media supplemented with caffeine or FdU, two groups of investigators have indicated that the number of common fragile sites may be much higher than previously recognized. Moreover, the number of these sites that coincide with chromosomal breakpoints in cancer cells was found to be greater than expected by chance.[29,30,70]

IDENTITY OF GENES LOCATED AT FRAGILE SITES

Although an association of fragile sites and cancer breakpoints has been recognized, the exact nature of the relationship at the DNA or gene level remains obscure. That human malignant diseases are characterized by nonrandom chromosomal abnormalities is now well established. A number of the genes located at the chromosomal break points have been identified, and the direct role of chromosome abnormalities in the pathogenesis of human neoplastic diseases is becoming increasingly evident. In many instances, the genes located at the breakpoints of specific rearrangements were previously recognized as proto-oncogenes; in several other instances, however, previously unknown transforming sequences have been identified. The cellular homologue of the transforming sequence of the Moloney murine sarcoma virus, c-*mos*, has been mapped to 8q22,[71] the breakpoint in the t(8;21) in AML. Another proto-oncogene, c-*ets*-1, has been mapped to 11q23 to q25.[72] This chromosome region is the breakpoint in the t(9;11) in AMoL as well as in the other translocations and deletions associated with this type of leukemia; it is also the breakpoint in the t(4;11) in ALL. Both 8q22 and 11q23 are the location of recognized heritable fragile sites (Fig 2). C-*erbB*, one of the transforming sequences associated with the avian erythroblastosis virus, has been mapped to the proximal region of 7p,[73] which also contains a fragile site as well as a breakpoint in a specific rearrangement, the t(1;7).[49] A transforming gene, *bcl-1*, was identified at the breakpoint on 11q13, which is involved in the t(11;14) in small-cell lymphoma [74] This band also contains a heritable fragile site; several cancer patients with rearrangements involving this band were found to be carriers of this fragile site.[42,67]

In addition to transforming sequences and proto-oncogenes, other genes have been mapped to chromosomal regions that are breakpoints in cancer cells and also are the locations of fragile sites. These genes include the α- and β-interferons (9p21 to p22, t(9;11) in AMoL, del(9p) or t(9p) in ALL).[75,76] In addition, Le Beau et al[77] have mapped the metallothionein family of genes to 16q22 and have shown that this gene cluster is split by the inv(16) and t(16;16) that occur in AMMoL. More importantly, Le Beau and Rowley[43] and Le Beau (unpublished observations, March 1985) have demonstrated that these patients were also carriers of a heritable fragile site at 16q22. With the use of molecular techniques, it will be possible to determine whether the fragile site in these patients also occurs within the metallothionein gene cluster and whether the fragile site involves precisely the same sequences as does the q22 breakpoint of the inv(16) noted in the leukemic cells of these patients. Moreover, it will also be feasible to clone and sequence this fragile site and to determine whether the putative common fragile site at 16q22 occurs at the same point at the DNA level as does the rarer, heritable fragile site.

If fragile sites serve as factors predisposing to chromosomal rearrangements in human neoplasia, one might expect that gene sequences integrally involved in neoplastic transformation would reside at these loci. Unfortunately, our knowledge about the genes located at fragile sites is still limited. In fact, although several genes are mapped to the region of fragile sites, no single gene has been identified at the precise site. The identification and determination of the normal cellular function of the genes located in regions that are both fragile sites and breakpoints in chromosomal rearrangements in malignant cells is an important goal in contemporary cancer research.

UNRESOLVED ISSUES

The reports of an association between the location of fragile sites and chromosomal break points in cancer cells raise a number of questions that have not been answered. Determining the baseline frequency of fragile sites in cancer patients and comparing this frequency to that in the general population is of primary importance. We do not yet know whether patients with cancer who have chromosome rearrangements other than inv(16), t(11;14), del(11q), or t(12;14) are also carriers of fragile sites in the altered chromosome regions. Are these fragile sites the rare heritable or the common fragile sites? Do fragile sites predispose individuals to cancer development? If so, which sites have such an effect, and to what extent? Is such a predisposition limited to the heritable fragile sites? In other words, is the risk associated with the heritable fragile sites different from that associated with the common fragile sites? Does a particular fragile site predispose to a specific type of cancer, and is this predisposition familial? For example, are there families carrying fragile sites in whom the same malignancy that is characterized by the same cytogenetic abnormality has developed in multiple members? If fragile sites are found in unaffected members of the family, how can this be explained? In such an event, can we identify the additional genetic or environmental factors that lead to the development of tumors in only some carriers of fragile sites? Are there additional classes of fragile sites whose expression can be induced? Can fragile sites serve to identify individuals at risk of developing cancer?

A major question is related to the identity of the genes located at fragile sites. Are these the genes that are affected by the chromosomal rearrangements in cancer cells? If so, this would indicate that the genes located at fragile sites are related to malignant transformation. This last question also is pertinent to another issue, namely, do fragile sites act in a specific way, or do they merely provide a mechanism that increases the frequency of breaks? If the gene at the fragile site is identical to the gene that is directly involved in cell transformation, then it will be relatively simple to clarify the association. If, on the other hand, fragile sites act in a relatively nonspecific way, essentially to increase the frequency with which a break occurs in one of two chromosome bands but not within the relevant DNA, then further chromosomal rearrangements must take place that elicit the critical genetic change. In this case, selection will act to favor the particular DNA rearrangement that leads to a proliferative advantage in a specific type of cell. For a proper understanding of this aspect of cellular transformation, it appears critical to determine the identity and the normal cellular function of the genes mapped to regions that are fragile sites as well as breakpoints in cancer cell chromosomes.

At present, the function and identity of genes located at the fragile sites remains an enigma. However, we know that some classes of fragile sites are inherited and that they may therefore be related to a genetic predisposition to the development of malignant diseases. In spite of the many unresolved issues, hematologists and oncologists should be aware of the research in this area because of its importance with regard to genetic susceptibility to cancer. Moreover, the goal of establishing the relationship between fragile sites and cancer breakpoints may enable us to develop means with which to anticipate, prevent, detect, or treat cancer more effectively.

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Macrophage Colony Formation Supported by Purified CSF-1 and/or Interleukin 3 in Serum-Free Culture: Evidence for Hierarchical Difference in Macrophage Colony-Forming Cells

By Kenichi Koike, E. Richard Stanley, James N. Ihle, and Makio Ogawa

Using a serum-free culture system, we examined murine macrophage colony formation from bone marrow cells cultured in the presence of purified CSF-1, interleukin 3 (IL 3) or a combination of the two factors. CSF-1 supported macrophage and neutrophil-macrophage colony formation, whereas IL-3 supported the formation of various types of single lineage and multilineage colonies. CSF-1 supported more macrophage colonies from bone marrow cells of normal mice than IL 3, whereas in cultures of bone marrow cells of 5-fluorouracil–treated mice, IL 3 supported more macrophage colonies. A combination of CSF-1 and IL 3 resulted in granulocyte-macrophage (GM) colony formation that was equal to or greater than the sum of GM colony formation supported by the factors individually. The combination of CSF-1 and IL 3 resulted in significant increases in the size of both macrophage and neutrophil-macrophage colonies. Similar increases in colony size were observed when CSF-1 was added to cultures five days after incubation of marrow cells with IL 3. These data support the concept that some of the macrophage colony–forming cells that respond to IL 3 are more primitive than those that are sensitive to CSF-1.

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CLONAL CULTURE ASSAYS for hematopoietic cells provide methods for studying functional properties of hematopoietic progenitors. The formation of colonies is supported by growth factors termed colony-stimulating factors (CSFs) that are released by a variety of blood and tissue cells.[1] Some CSFs have been purified to homogeneity and/or molecularly cloned.[2–7] Studies of the purified factors in clonal culture suggest that formation of some types of single-lineage colonies can be supported by more than one factor. For example, CSF-1,[2] GM-CSF,[7] and interleukin 3 (IL 3)[8–11] have been reported to support macrophage colony formation in culture. CSF-1 is relatively specific in stimulation of macrophage colony formation by mouse bone marrow cells,[12] whereas IL 3 supports the formation of a wide variety of single and multilineage colonies.[8–11]

It is generally held that the majority of macrophage colonies in culture are derived from monopotent macrophage progenitors. In analysis of murine paired progenitors, homologous pairs of macrophage colonies were often observed.[13,14] This observation was consistent with the derivation of macrophage colonies from monopotent macrophage progenitors. In the same analysis of paired progenitors, however, we also observed pairs consisting of a macrophage colony and a multilineage colony. This data suggested that committed macrophage progenitors may be derived directly from multipotent progenitors.

It is possible that different CSFs support macrophage progenitors differing in hierarchical stages. In this paper, we wished to delineate further the roles of IL 3 and CSF-1 in macrophage colony formation in culture. To improve the specificity, we established a serum-free culture system and studied macrophage colony formation from marrow cells obtained from normal and 5-fluorourcil (5-FU)-treated mice in the presence of CSF-1 and/or IL 3. 5-FU was used to obtain a population enriched for primitive progenitors. The results suggest that the macrophage colony–forming cells that respond to IL 3 may be more primitive than the macrophage progenitors that are dependent on CSF-1.

MATERIALS AND METHODS

Pure CSF-1 and IL 3. CSF-1 (~8 × 10^7 U/mg protein) was purified to homogeneity from serum-free mouse L cell–conditioned medium as described previously.[2,15] The activity in units per milliliter of CSF-1 preparations was obtained by comparing its activity with a standard preparation in a bone marrow colony assay in agar as described previously.[12] The stock preparation of CSF-1 containing 20,000 U/mL activity was kept frozen in α-medium containing 0 1% crystallized bovine serum albumin (BSA, Sigma Chemical Co, St Louis). IL 3 (~5 × 10^6 U/mg protein) had been purified to homogeneity as previously described.[16] Thymidine incorporation by the IL 3–dependent cell line FDC-P1 was used for the measurement of IL 3 activity [16] The stock solution containing 40,000 U/mL was kept frozen in phosphate-buffered saline containing 1% BSA.

Cell preparation. Ten- to 15-week-old female BDF_1 mice were obtained from Simonsen's Laboratory, Gilroy, Calif. Single-cell suspensions were prepared from pooled femurs of three to five mice. 5-FU (Adria, Laboratories, Inc, Columbus, Ohio) was administered intravenously through the tail vein of the mice at a dose of 150 mg/kg body weight. Bone marrow cells were harvested four days after 5-FU injection.

Clonal cell cultures. Serum-free clonal culture was carried out in 35-mm Lux suspension culture plates (5221R, Flow Laboratories, Inc, Rockville, Md) by using a modification of the technique described by Iscove et al.[17] Unless otherwise specified, culture consisted of 4 × 10^4/mL marrow cells from normal mice or 2 × 10^4/mL marrow cells from 5-FU–treated mice, α-medium (Flow),

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1.2% 1,500 cp methylcellulose (Fisher Scientific Co, Norcross, Ga), 1% crystallized globulin-free deionized BSA (Sigma), 10^{-4} mol/L mercaptoethanol (Eastman Organic Chemicals, Rochester, NY), 300 μg/mL fully iron-saturated human transferrin (approximately 98% pure, Sigma), 160 μg/mL soybean lecithin (Sigma), 96 μg/mL cholesterol (Sigma), 10^{-7} mol/L sodium selenite (Sigma), and 200 U/mL IL 3 or 200 U/mL CSF-1. The standard cultures contained 30% fetal bovine serum (Flow) instead of a combination of transferrin, lecithin, cholesterol, and sodium selenite. Plates were incubated at 37 °C in a humidified atmosphere flushed with 5% CO_2 in air.

In situ determination of colony types. In standard experiments, colony types were determined by in situ observation on an inverted microscope. In this case, colony types were classified as to whether they contained granulocyte, macrophage, megakaryocyte, and/or mast cell lineages. GM colonies containing more than 50 cells were scored between days 8 and 14 as indicated in each experiment. Unless otherwise specified, megakaryocyte colonies consisting of more than four cells were scored on day 8 of incubation, granulocyte-macrophage-megakaryocyte (GMM) colonies on day 12 of culture[18] and mast cell colonies and blast cell colonies on day 16 of incubation.[19]

Cytologic examination of colonies. In some experiments, colony types were determined by differential counting of May-Grünwald-Giemsa–stained preparations. We reported previously that the phenotypes of colonies containing blast cells may continue to change as these colonies mature in culture.[18] In serial observations of colony formation from spleen cells of 5-FU–treated mice, we also observed continued appearance of new macrophage and multilineage colonies and their subsequent development throughout the incubation up to 16 days.[20] To determine the full potentials of colonies supported by CSF-1 or IL 3, we carried out cytologic examination of colonies that were harvested from mapping studies. We plated 8 × 10^4 bone marrow cells from normal mice and 4 × 10^4 cells from 5-FU–treated mice in the presence of CSF-1 or IL 3 and carried out daily examination of plates on an inverted microscope. When a new colony appeared, its location in the plate was recorded and subsequent growth and differentiation followed. When a colony appeared to have matured and did not change its morphology or size for three days, it was lifted from the methylcellulose medium by use of an Eppendorf micropipette, and differential counting carried out on May-Grünwald-Giemsa–stained smears. Routinely, differential counts were done on 500 cells. When indicated, colonies were individually lifted by an Eppendorf micropipette, made into single-cell suspensions, and divided into two aliquots. One half of the sample was used for counting in a counting chamber and the other half for analysis of differential counts. Abbreviations used for the description of lineages are modifications of those recommended at a workshop of a UCLA symposium[21]: n, neutrophil; m, macrophage; mast, mast cell; e, eosinophil; E, erythrocyte, M, megakaryocyte.

RESULTS

Colonies supported by CSF-1 or IL 3 in serum-free culture. CSF-1 supported only GM colonies whereas cultures containing IL 3 revealed various types of colonies. GM colony formation seen in serum-free culture in the presence of 200 U/mL CSF-1 or 200 U/mL IL 3 was approximately one half to two thirds of the colony number in the standard culture conditions. An example of such a comparison is presented in Table 1. Although a few colonies were present in serum-containing culture without IL 3 or CSF, there were no colonies in the serum-free plates without added factors. Results of cytologic analyses of all colonies supported by these factors in serum-free culture are presented in Table 2. The majority of the colonies seen in cultures with CSF-1 were small macrophage colonies consisting of less than 400 cells, whereas the remaining one third were neutrophil-macrophage colonies. CSF-1 did not support pure neutrophil colonies. Similar to our previous observations in standard culture conditions,[9-11] IL 3 supported growth of single as well as multilineage colonies revealing varying combinations of lineages in a serum-free system. Macrophage colonies were small and consisted of mature macrophages.

Table 1. GM Colony Formation by Bone Marrow Cells of Normal Mice in Serum-Free or Serum-Containing Cultures

Factor	Serum-Free Culture	Serum-Containing Culture
CSF-1	56 ± 11	111 ± 14
IL 3	58 ± 4	97 ± 7
	(73 ± 6)*	(103 ± 6)
None	0	6 ± 3

*The data presented in parentheses indicate the number of all types of colonies supported by IL 3.

Bone marrow cells in 4 × 10^4 cells/mL were cultured in the presence of 200 U/mL CSF-1 or 200 U/mL IL 3, and colonies were counted on day 8.

Data represent the mean ± SD of quadruplicate cultures.

Next, we carried out a serial examination of colony formation on an inverted microscope to determine the optimal period of culture for neutrophil and/or macrophage colony formation by the purified factors. Since it is not always possible to distinguish accurately among pure macrophage colonies, neutrophil-macrophage colonies, and neutrophil colonies in situ, we combined all three types and reported the total number of GM colonies. Plateaus for the numbers of GM colonies were seen between day 8 and day 11 of incubation for both CSF-1 and IL 3.

Dose response studies. We then examined the effects of various concentrations of CSF-1 and IL 3 on colony formation by normal bone marrow cells. Both factors in serial

Table 2. Analysis of Types of Colonies Derived From Marrow Cells of Normal Mice That Are Serially Harvested From Serum-Free Cultures Containing CSF-1 or IL 3

Lineage Expressed in the Colonies						
n	m	mast	e	M	CSF-1	IL 3
+					0	30
	+				48	21
		+			0	5
				+	0	3
+	+				28	50
	+	+			0	2
	+			+	0	1
		+		+	0	5
+	+	+			0	1
+	+		+		0	1
	+	+		+	0	2
+	+	+		+	0	1
Totals					76	122

Numbers of colonies derived from 8 × 10^4 cells in four dishes.

Table 3. Effects of Various Concentrations of CSF-1 and IL 3 on Colony Formation by Bone Marrow Cells of Normal Mice

Concentration (U/mL)	CSF-1	IL 3					
	GM	GM	M	GMM	MAST	Bl	Total
400	51 ± 9	61 ± 6	2 ± 1	5 ± 1	6 ± 1	2 ± 1	76 ± 7
200	53 ± 5	58 ± 4	3 ± 1	3 ± 1	5 ± 2	4 ± 1	73 ± 6
100	27 ± 3	40 ± 4	2 ± 1	1 ± 1	5 ± 1	2 ± 1	50 ± 5
50	8 ± 3	23 ± 6	1 ± 1	2 ± 1	6 ± 3	2 ± 2	34 ± 10
25	0	14 ± 4	1 ± 1	0	3 ± 2	2 ± 2	20 ± 5
12.5	0	8 ± 2	0	0	2 ± 1	2 ± 1	12 ± 2
6.3	0	3 ± 1	1 ± 1	0	1 ± 2	1 ± 1	6 ± 2
3.1	0	2 ± 1	0	0	0	0	2 ± 1
1.6	0	1 ± 1	0	0	1 ± 1	0	2 ± 1
0.8	0	1 ± 1	0	0	0	0	1 ± 1

Data represent the mean ± SD of quadruplicate cultures containing 4 × 10^4 cells/dish.

Abbreviations: M, megakaryocyte colonies; MAST, mast cell colonies; Bl, blast cell colonies.

dilutions were added to methylcellulose culture of normal bone marrow cells, and the colonies were counted using in situ identification. The results of the dose-response studies are presented in Table 3. Maximal GM colony stimulation was seen at 200 U/mL of CSF-1 or IL 3. Based on these studies, the standard concentrations for CSF-1 and IL 3 became 200 U/mL.

Effects of combination of CSF-1 and IL 3. Results presented in Tables 1 to 3 clearly demonstrate that macrophage colony formation is supported by both CSF-1 and IL 3. In addition, multilineage colony formation was supported by IL 3, as was documented previously.[9-11] The latter information suggests that the targets of IL 3 include primitive progenitors. To better define the roles of CSF-1 and IL 3, we cultured marrow cells in the presence of CSF-1 or IL 3 and the combination of CSF-1 and IL 3 and examined the number, types, and sizes of GM colonies.

First, we cultured 3 × 10^4 marrow cells of normal mice and 2 × 10^4 marrow cells of 5-FU–treated mice in quadruplicate cultures and analyzed GM colonies on days 8, 11, and 14 of incubation (Table 4). The number of GM colonies supported by a combination of CSF-1 and IL 3 were close to or in excess of the sum of the number of colonies supported by CSF-1 and IL 3 separately. This information suggests that the GM colony–forming cells that are targets of CSF-1 and IL 3 are not identical. They could represent basically separate populations without significant overlap. Alternatively, there may be populations of GM progenitors that require both CSF-1 and IL 3 for their proliferation.

In addition to the increase in the number of colonies, the combination of CSF-1 and IL 3 resulted in a significant enlargement of GM colonies. In a separate experiment, 20 macrophage and 20 neutrophil-macrophage colonies were individually picked from each group and analyzed for their cell number and composition. The size distribution of these colonies is presented in Fig 1 on a logarithmic scale. For both macrophage and neutrophil-macrophage colonies, the populations grown in the presence of both CSF-1 and IL 3 were significantly larger than colonies grown either in CSF-1 or IL 3 alone. Addition of CSF-1 did not increase the size of neutrophil colonies supported by IL 3 (data not shown).

Delayed addition of factors. The aforementioned data suggested that one of the two factors promoted the growth of the colonies initiated by the other factor. IL 3 is known to exert its effects on primitive multipotent progenitors. Therefore, we entertained the possibility that CSF-1 promoted the development of IL 3–initiated macrophage colonies. To test this premise, we carried out delayed addition experiments. In group A, bone marrow cells from normal mice were plated in the presence of 200 U/mL IL 3. Five days later, either 200 units of CSF-1 or α-medium was layered over each dish, and culture was continued. Six days later, all GM colonies were scored in situ and individually picked for simultaneous cytologic examination and estimation of colony size. The mean and one SD of the numbers of GM colonies derived from 3 × 10^4 cells were 59 ± 1 and 46 ± 6 for CSF-1 and α-medium groups, respectively. These numbers were significantly different ($P < .05$) according to Student's t test and suggested the presence of GM progenitors that require both CSF-1 and IL 3 for continued proliferation in culture.

The delayed addition of CSF-1 not only increased the number of GM colonies but also resulted in a significant enlargement of some of the GM colonies. Analysis of colony size of 20 randomly chosen macrophage colonies and neutrophil-macrophage colonies in the two experimental groups is presented in Fig 2A. Both macrophage colonies and neutro-

Table 4. Effects of CSF-1 and/or IL 3 on GM Colony Formation by Bone Marrow Cells of Normal and 5-FU–Treated Mice

Day of Observation	Normal Mice			5-FU–Treated Mice		
	CSF-1	IL 3	CSF-1 + IL 3	CSF-1	IL 3	CSF-1 + IL 3
Day 8	45 ± 4	45 ± 5	103 ± 8	4 ± 2	2 ± 1	11 ± 2
Day 11	42 ± 4	52 ± 4	93 ± 8	4 ± 2	5 ± 2	14 ± 1
Day 14	—	—	—	3 ± 2	11 ± 2	19 ± 3

Data represent the mean ± SD of quadruplicate plates containing 3 × 10^4 marrow cells from normal mice or 2 × 10^4 cells from 5-FU–treated mice

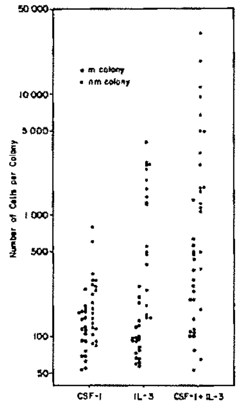

Fig 1. Distributions of sizes of 20 randomly chosen macrophage (m) colonies and 20 neutrophil-macrophage (nm) colonies cultured in the presence of CSF-1 or IL 3 or a combination of CSF-1 and IL 3. Macrophage colonies in the CSF-1 + IL 3 group were larger than those in the CSF-1 group ($P < .01$) and the IL 3 group ($P < .01$). Neutrophil-macrophage colonies in the CSF-1 + IL 3 group were larger than those in the CSF-1 group ($P < .001$) and the IL 3 group ($P < .02$). Statistical analyses were done using Student's *t* test on logarithms of the cell numbers.

phil-macrophage colonies in the CSF-1 group were significantly larger than colonies in the α-medium group. The increase in the size of neutrophil-macrophage colonies was due to an increase in the macrophage population ($P < .001$ by *t* test on logarithms of the cell number) but was not due to an increase in neutrophils ($P > .1$). The data for the CSF-1 group in Fig 2A are similar to the data for the group with a combination of IL 3 and CSF-1 in Fig 1, suggesting that CSF-1 promotes cell proliferation during later stages of macrophage colony development that had been initiated by IL 3.

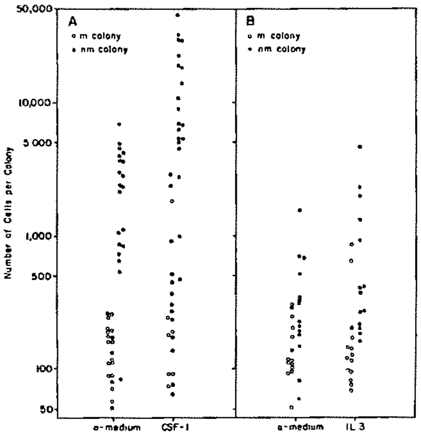

Fig 2. Distributions of sizes of macrophage colonies (m) and neutrophil-macrophage (nm) colonies seen in the delayed addition experiment. (A) Cultures were initiated with IL 3 on day 0. CSF-1 or α-medium was added on day 5. Both macrophage colonies and neutrophil-macrophage colonies in cultures receiving CSF-1 on day 5 of incubation were larger than their counterparts in the control (α-medium) group, $P < .02$ and $P < .001$, respectively ($n = 20$). (B) Cultures of bone marrow cells were initiated with 200 U/mL of CSF-1 and received 200 units of IL 3 or α-medium on day 5. There was no significant difference in the size of colonies between experimental (IL 3) and control (α-medium) groups ($n = 14$). Student's *t* test was applied to logarithms of the cell numbers as was in Fig 1.

Cultures in group B received the factors in reverse order. Bone marrow cells were plated in the presence of 200 U/mL CSF-1 and five days later received either 200 units of IL 3 or α-medium. When colonies were counted six days thereafter, the mean and one SD of the number of GM colonies from 3×10^4 marrow cells were 28 ± 5 and 22 ± 4 for the IL 3 and α-medium groups, respectively, and did not show significant differences ($P > .1$ by the *t* test). The size of GM colonies did not differ significantly between the experimental and control groups. Analysis of colony size of 14 randomly picked macrophage colonies and neutrophil-macrophage colonies is presented in Fig 2B. Colonies in both groups were relatively small, and none reached the size of the large colonies shown in Figs 1 and 2A.

Effects of CSF-1 and IL 3 on colony formation from bone marrow cells of 5-FU–treated mice. Data presented in Fig 2 suggested that the macrophage colony–forming cells that responded to CSF-1 are maturer than those that are targets for IL 3. Additional support for the heterogeneity of macrophage CFU was derived from studies of colony formation from bone marrow cells of 5-FU–treated mice. As originally described by Hodgson and Bradley[22] and subsequently in our laboratory,[20] an intravenous injection of 150 mg/kg of 5-FU into mice selectively kills cells that have active DNA synthesis while sparing dormant primitive hematopoietic stem cells. We cultured bone marrow cells of 5-FU–treated mice in the presence of CSF-1 or IL 3. Table 5 presents the cytologic examination of all colonies that were sequentially identified in plates containing either CSF-1 or IL 3. Only three small macrophage colonies and no neutrophil-macrophage colonies were seen in plates containing CSF-1. In contrast, during 16 days of incubation, a total of nine macrophage colonies and 23 neutrophil-macrophage colonies were observed in plates containing IL 3. Therefore, the number of macrophage and neutrophil-macrophage colonies supported by CSF-1 in

Table 5. Analysis of Types of Colonies Derived From Marrow Cells of 5-FU–Treated Mice That Are Serially Harvested From Serum-Free Cultures Containing CSF-1 or IL 3

Lineage Expressed in the Colonies							
n	m	mast	e	E	M	CSF-1	IL 3
+						0	5
	+					3	9
		+				0	1
					+	0	6
+	+					0	23
+					+	0	1
	+	+				0	1
+	+	+				0	8
	+	+			+	0	3
+	+	+	+			0	2
+	+	+			+	0	6
+	+	+	+		+	0	3
+	+	+	+	+	+	0	1
Totals						3	69

Numbers of colonies derived from 4×10^4 cells in two dishes.

post–5-FU marrow cells was significantly smaller than that of those supported by IL 3. This result markedly contrasted the result of culture of normal marrow cells presented in Table 2 and provided additional evidence that IL 3–dependent macrophage proliferation represents an earlier stage of differentiation than the CSF-1–dependent process.

DISCUSSION

As previously indicated, the development of single-lineage colonies may be supported by more than one factor. Bradley and his associates[22-24] identified a primitive macrophage progenitor termed HPP-CFC and proposed that the development of large macrophage colonies from HPP-CFC requires both CSF-1 and a factor(s) with synergistic activity. The synergistic activity is present in media conditioned by human spleens and placenta or the supernatant of cultures of the murine myelomonocytic leukemia cell line WEHI-3. Iscove et al[25] also observed synergistic activity in support of macrophage colony formation by partially purified WEHI-3 burst-promoting activity (BPA) and CSF-1. Although the synergistic activity of WEHI-3 is likely to be due to IL 3,[26] the synergistic activity in human preparations has different properties.[27]

In this paper, we addressed the mechanisms of synergism between CSF-1 and IL 3 on macrophage colony formation in culture. Since fetal bovine serum is potentially an endogenous source of various types of CSF, we carried out studies in a serum-free culture system. Although this system is not chemically defined, it provided a system in which macrophage colony formation was totally dependent on added growth factors. Using purified preparations of CSF-1 and IL 3, we have shown that CSF-1 supports more macrophage colonies from bone marrow cells of normal mice than IL 3, whereas in cultures of bone marrow cells of 5-FU–treated mice, IL 3 supported more macrophage colonies. A significant increase in the size of macrophage colonies and neutrophil-macrophage colonies was observed when CSF-1 was added to cultures five days after incubation of marrow cells with IL 3. The increase in the size of neutrophil-macrophage colonies was due to an increase in the macrophage population. These data suggested the heterogeneity in macrophage colony–forming cells that respond to IL 3 and CSF-1 and provided further clarification of the synergistic mechanisms between CSF-1 and IL 3 on macrophage colony formation.

Studies of the action of CSF-1 and IL 3 in suspension cultures also indicated that IL 3 acts on more primitive progenitors than CSF-1.[28] In cultures enriched for very primitive cells (bone marrow cells harvested one day after injection of 5-FU), IL 3 increased the number of nonadherent CSF-1 binding cells whereas CSF-1 did not. In cultures containing more mature cells (marrow cells harvested six days after 5-FU injection), CSF-1 generated approximately 17 times more adherent CSF-binding cells than partially purified IL 3. In addition, the effects of IL 3 plus CSF-1 on the very primitive cells were shown to be slightly synergistic. These observations on CSF-1–binding cells in suspension culture corroborate our current studies on macrophage colony–forming cells and indicate the heterogeneity in macrophage progenitors that are responsive to CSF-1 and IL 3.

Mechanisms of some of the observations made in the current studies are unclear. Purified CSF-1 supported the formation of macrophage colonies and neutrophil-macrophage colonies, but not pure neutrophil colonies. We observed a few neutrophil colonies supported by CSF-1 in serum-containing culture (data not shown). Using a standard culture system containing serum and a different strain of mice, we have observed no neutrophil colony production by CSF.[29] Metcalf and Burgess[30] observed the presence of small numbers (2%) of neutrophil colonies. Differentiation of neutrophils requires neutrophil specific factor(s)[31] Small amounts of such factor(s) may be present in fetal bovine serum and may be released by macrophages in the neutrophil-macrophage colonies. Purified IL 3 supported the formation of single-lineage neutrophil, macrophage, mast cell, and megakaryocyte colonies. The majority of the cells in these colonies are mature cells with distinct cytoplasmic differentiation.

In our previous publication,[11] we presented evidence that IL 3 provides a permissive milieu for the growth of multipotential progenitors that begin active proliferation after varying periods of G_0. We have also shown that cells that are committed to single-lineage differentiation may be derived directly from multipotential progenitors by a stochastic process.[13] It appears that the stochastic process of commitment to single lineages is supported by IL 3. As these progenitors mature, they appear to develop sensitivity to more lineage-restricted factors such as CSF-1. In the case of CSF-1, the development of sensitivity is correlated to an increase in the expression of CSF-1 receptors.[28]

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Myeloperoxidase Biosynthesis by a Human Promyelocytic Leukemia Cell Line: Insight Into Myeloperoxidase Deficiency

By William M. Nauseef

The biosynthesis and processing of myeloperoxidase (MPO), a cationic enzyme present in the azurophilic granules of human polymorphonuclear leukocytes (PMNs), were studied in the human promyelocytic leukemia cell line, HL-60. HL-60 cells produce large quantities of enzymatically active MPO that has the same electrophoretic behavior as MPO isolated from normal PMNs. Mature MPO is a glycoprotein of approximately 150,000 molecular weight (mol wt) composed of two heavy-light protomers ($\alpha_2\beta_2$) with subunits of 59,000 and 13,500 mol wt, respectively, under reducing conditions. The primary translation product of MPO messenger RNA (mRNA) isolated from HL-60 cells was a single polypeptide of mol wt 80,000. In HL-60 cells labeled with [^{35}S]-methionine, the labeled MPO isolated by immunoprecipitation had a mol wt of 89,000. Treatment of this 89-kilodalton (kDa) species with endoglycosidase H produced a 79-kDa peptide, suggesting that the 89-kDa protein contained high-mannose side chains. The 89-kDa species had no detectable peroxidase activity. During chase experiments some of the 89-kDa peptide was processed to smaller species of mol wt 39,000, 59,000, and 13,500, although a fraction of the 89-kDa peptide remained unprocessed after a chase of 100 hours. In addition, a small amount of the 89-kDa peptide appeared in the medium without any of the processed smaller peptides. These studies suggest (1) that the primary translation product in MPO biosynthesis is an 80-kDa peptide that undergoes cotranslational cleavage of the signal peptide and glycosylation to produce an 89-kDa pro-MPO, (2) that pro-MPO is a single polypeptide containing the α and β subunits of MPO and contains endoglycosidase H–susceptible high-mannose side chains, and (3) that posttranslational modification of pro-MPO results in targeting to the lysosome and proteolytic maturation of pro-MPO to active enzyme. In light of the previous observation that MPO-deficient and normal PMNs contain an 89-kDa protein immunochemically related to MPO, these studies on MPO biosynthesis indirectly support the hypothesis that defective posttranslation processing by pro-MPO may underlie hereditary MPO deficiency.

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MYELOPEROXIDASE (MPO),[1] a cationic glycoprotein present in the azurophilic granules of human polymorphonuclear leukocytes (PMNs), plays an important role in PMN microbicidal activity.[1] Numerous investigators have characterized the function of MPO in oxygen-dependent events following PMN stimulation. The structure of MPO has likewise been extensively studied,[2] and although some disagreement exists, most studies suggest that MPO is composed of a pair of identical heavy-light protomers with a combined mol wt of approximately 140 to 150,000.[3-9] The two protomers are each composed of a 59-kDa α subunit and a 13.5-kDa β subunit and are linked along their long axis by a single disulfide bond joining the two heavy[5] or two light[6] subunits. MPO contains 2% to 4% carbohydrate,[6,10] and a recent study of the subunit structure indicates that the 59-kDa subunit is glycosylated, whereas the 13.5-kDa subunit has no carbohydrate.[11]

Hereditary MPO deficiency has been identified as a relatively common defect, present in one of 2,000 individuals in the population.[12] Although initial studies suggested that MPO deficiency is the result of a structural gene defect inherited as an autosomal recessive trait,[13-18] recent family studies suggest that the mode of inheritance is more complex.[12] In vitro studies of PMN function from such individuals demonstrate delayed bactericidal[12,13,19-21] and an absence of candidacidal action.[12-14,22] PMNs from individuals with functional MPO deficiency do not contain normal subunits of MPO, as determined by polyacrylamide gel electrophoresis (PAGE) and immunoblotting.[3] However, both MPO-deficient and normal PMNs contain an 89-kDa peptide that is immunochemically related to MPO.[3] The presence of this large peptide immunochemically related to MPO in both MPO-deficient and normal PMNs suggests that MPO deficiency may be due to defective processing of an MPO precursor, which results in aberrant packaging of MPO into the azurophilic granules. The goals of this work are to characterize the events in MPO synthesis and to determine the steps that are critical for correct transfer of nascent MPO from its intracellular site of synthesis to its final intracellular destination in the azurophilic granule. With these results it will be possible to examine the nature of the defect in hereditary MPO deficiency.

For these studies the human promyelocytic leukemia cell line HL-60[23,24] was used to examine MPO synthesis because PMNs are terminally differentiated cells with little, if any, protein synthesis.[25] Undifferentiated HL-60 cells produce 47.5 μg of MPO per 10^7 cells,[26] and the MPO produced has a normal subunit structure as determined by immunoblot analysis.[27] This cell line thus provides a useful system for the study of MPO synthesis. Data presented here are consistent with the hypothesis that MPO deficiency is due not to the absence of the gene for MPO synthesis but to a defect in

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cotranslational or posttranslational processing of the precursor protein of MPO, resulting in a failure to package the enzyme correctly into the azurophilic granule.

MATERIALS AND METHODS

Reagents. Diisopropylfluorophosphate (DFP), leupeptin, pepstatin A, phenylmethylsulfonylfluoride (PMSF), cetyltrimethylammonium bromide (CETAB), Triton X-100, EDTA, and molecular weight markers for polyacrylamide gels were obtained from Sigma Chemical Co (St Louis) Suspensions of formalin-fixed *Staphylococcus aureus* (Cowan strain) were used as a source of protein A in immunoprecipitation and were obtained from Bethesda Research Laboratories (Gaithersburg, Md). Cowan cells were heated to 95 °C for 30 minutes in 10% 2-mercaptoethanol and 3% sodium dodecyl sulfate (SDS) as previously described[28] and stored as 10% suspensions in phosphate-buffered saline at −20 °C until use. Immediately prior to use, the 10% suspension of Cowan cells was washed sequentially with 1-mL volumes of 0.5% Triton X-100 (Tx-100) in 10 mmol/L Tris buffer, pH 7.5, with 150 mmol/L NaCl (TBS), 2 mol/L urea in .5% Tx-100 in TBS, 1 mg/mL bovine serum albumin in 0.5% Tx-100 in TBS, and TBS before reconstitution to 10% suspension in TBS. RPMI 1640 Select Amine Kit (GIBCO, Grand Island, NY) was used to prepare the methionine-free medium that was supplemented with 1 mmol/L pyruvate and 10% heat-inactivated fetal calf serum (FCS) supplied by the Tissue Culture Facility of the Cancer Center at the University of Iowa. [^{35}S]-methionine of high specific activity (1,495 Ci/mmol) was purchased from Amersham Corp (Arlington Heights, Ill). Endoglycosidase H was obtained from Dr Frank Maley (Albany, NY). Micrococcal nuclease (15,000 U/mL) was obtained from Worthington Biochemical Corp (Freehold, NJ)

HL-60 cells. The human promyelocytic cell line HL-60 was obtained from Dr Harry L. Malech of Yale University and grown in RPMI 1640 medium supplemented with 1 mmol/L pyruvate and 10% heat-inactivated FCS.

Polyacrylamide gel electrophoresis Samples were solubilized in gel sample buffer and placed in a boiling water bath for two minutes. Bromophenol blue tracking dye in 50% glycerol was added to samples prior to electrophoresis. Electrophoresis of samples from labeling experiments or in vitro translations was performed in 0.7-mm-thick gradient gels of 8% to 14% acrylamide and 0% to 14% glycerol with a stacking gel of 3.5% acrylamide. Gels were electrophoresed overnight at 40 V (constant voltage), and the voltage was increased the following morning until the dye front migrated 10.5 cm into the gel. The gel was then stained for protein with R-250 Coomassie blue in 50% methanol and 10% acetic acid and destained in 15% methanol/10% acetic acid. For fluorography the gel was soaked in 400 mL (20 vol) water for 30 minutes and 200 mL (10 vol) of 1 mol/L salicylate (pH 5.7) for 30 minutes as described by Chamberlain.[29] Gels were dried and exposed to preflashed X-Omatic XAR-5 film (Kodak, Rochester, NY) and kept at −80 °C until the autoradiograph was developed. Molecular weights were calculated from plots of R_f v log M_r, using myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (BSA, 66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) as standards.

Immunoautoradiography. Samples were electrophoresed into 10% polyacrylamide gels prior to electroblotting to nitrocellulose paper according to the method of Towbin et al[30] and processed as described previously [3] The nitrocellulose paper to which proteins had been transferred was exposed sequentially to 3% BSA in Burridge buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, .005% NaN$_3$, pH 7.4), anti-MPO antiserum diluted 1:40 in 3% BSA–Burridge buffer, and [^{125}I]-protein A (200,000 cpm/mL) in 3% BSA–Burridge buffer. After multiple washes in Burridge buffer, the paper was dried and exposed to X-Omatic film in a casette with an intensifying screen.

Preparation of RNA Total cellular RNA was prepared by the method of Chirgwin et al[31] from pellets of HL-60 cells harvested from cultures in suspension. The total RNA obtained, estimated by the absorbance at 260 nm (A_{260} = 1 for 42 μg/mL), was 3.05 μg per 10^6 cells Later experiments using a phenol-chloroform extraction of HL-60 cell pellets gave qualitatively the same results but a higher yield of biologically functional RNA as judged by in vitro translation.

In vitro protein synthesis The RNA isolated from HL-60 cells was translated in vitro according to the method of Hunt and Jackson[32] using a rabbit reticulocyte-lysate system and [^{35}S]-methionine. Lysates of rabbit reticulocytes were prepared in the laboratory of Dr Edward C. Heath, Department of Biochemistry, University of Iowa. Lysates were prepared without addition of exogenous methionine and treated with micrococcal nuclease[33] to inhibit translation of endogenous RNA and to increase dependence of translation on exogenously added RNA. Typical translations were done in a volume of 55 μL and contained 5 to 15 μg of total RNA and 85 to 100 μCi of [^{35}S] methionine. In all experiments translation mixtures to which no RNA was added served as controls.

Precipitations Protein was precipitated from 10% trichloroacetic acid and counted to estimate the total amount of [^{35}S]-methionine incorporated into protein. Immunoprecipitations were carried out in two steps: the first to remove any labeled proteins that nonspecifically bind to rabbit serum or to Cowan cells and the second to precipitate specifically labeled protein related to MPO. Samples were diluted in a microfuge tube to 150 μL with TBS, 7 μL of nonimmune rabbit serum was added, and the sample was incubated at 4 °C for 30 minutes. Cowan cells (100 μL of a washed 10% suspension) were added and the sample vortexed and incubated at 4 °C for 30 minutes Cowan cells were removed by centrifugation (12,000 g for one minute) and the supernatant fluid (approx 230 μl) transferred to another 1.5-mL microfuge tube The sample was brought to 2% SDS by the addition of 35.4 μL of 15% SDS, placed in a boiling water bath for two minutes, and then diluted with 1.06 mL (4 vol) of dilution buffer (50 mmol/L Tris-HCl 190 mmol/L NaCl, 2.5% Tx-100, 6 mmol/L EDTA, pH 7.4). The sample was vortexed, 5 μL of rabbit anti-MPO antiserum prepared as described previously[3] added, and the sample incubated at 4 °C for five to 16 hours Cowan cells (100 μL of a 10% suspension) were added and the sample incubated at 4 °C for 30 minutes The suspension was layered atop a 400-μL cushion of 1 mol/L sucrose in TBS and centrifuged (12,000 g for five minutes in a Beckman Microfuge 12) to pellet the Cowan cells The supernatant fluid was aspirated and discarded and the pellet washed sequentially with 1 mL of 0.5% Tx-100/TBS, 2 mol/L urea in 0.5% Tx-100/TBS, 1 mg/mL BSA in 0.5% Tx-100/TBS, and TBS. After the final wash the pellet was resuspended in 100 μL of gel sample buffer (62.5 mmol/L Tris HCl, 190 mmol/L NaCl, 6 mmol/L EDTA, 2.3% SDS, 2.5% Tx-100, 5% 2-mercaptoethanol, pH 6.8), heated for two minutes in a boiling water bath to release the antigen-antibody complex from the protein A on the Cowan cells, vortexed while hot, and centrifuged to remove Cowan cells A 20-μL aliquot of the supernatant fluid was solubilized in 200 μL Scintigest (Fisher Chemical Co, St Louis) and 2.5 mL of scintillation fluid (19 g 2,5-diphenyloxazole, 439 mg dimethyl-2,5-diphenyloxazole, 1,165 mL Tx-100 in 2,330 mL toluene) to count activity of [^{35}S]. The remainder of the sample was electrophoresed into an SDS polyacrylamide gel.

Separation of subcellular components. HL-60 cells were harvested, washed, and resuspended in 20 mL of relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 1 mmol/L adenosine triphosphate (ATP) (Na)$_2$, 3.5 mmol/L MgCl$_2$, 10 mmol/L piperazine-N,N^1-bis

(2-ethanesulfonic acid) (PIPES), pH 7.3, containing 1 mmol/L DFP for 30 minutes on ice as described previously for PMNs.[34] The cells were centrifuged (1400 g for ten minutes) and resuspended in the same buffer without DFP. The subcellular fractions were obtained by nitrogen cavitation separated on a discontinuous gradient of Percoll according to the technique of Borregaard et al.[34] Fractions (1.0 mL) were collected and Percoll removed by ultracentrifugation (180,000 g for 120 minutes) prior to further analysis.

Biochemical analysis. Fractions from the Percoll gradient were assayed for biochemical markers of subcellular organelles. Alkaline phosphatase (plasma membrane) activity was assayed in a 1-mmol/L $MgCl_2$, 50-mmol/L sodium barbital buffer (pH 10.5) as the hydrolysis of *p*-nitrophenyl phosphate (1 mg/mL).[35] Lysozyme (granules) was assayed spectrophotometrically at 450 nm as the disappearance of turbidity of 0.2 mg/mL of *Micrococcus lysodeikticus* in 67 mmol/L sodium phosphate buffer at pH 6.2[36] Myeloperoxidase activity was determined by the oxidation of *o*-dianisidine.[37] Galactosyl transferase (Golgi) was measured as described by Beaufay et al[38] and modified by Brown and Swank.[39]

Biosynthetic labeling. HL-60 cells were harvested by centrifugation and resuspended at 3×10^6/mL in a methionine-free RPMI 1640 medium with 1 mmol/L pyruvate and 10% heat-inactivated FCS that had been dialyzed against phosphate-buffered saline (pH 7.4) in 6,000-mol wt cut-off dialysis tubing prior to addition to the RPMI. Cells were incubated for 30 minutes at 37 °C in an atmosphere of 5% CO_2 in methionine-free medium prior to addition of $[^{35}S]$-methionine (100 μCi/mL). In pulse-chase experiments an excess of unlabeled methionine (1 mmol/L) was added at the end of the labeling interval, and incubation was continued for the designated period of time. At the end of the incubation, cells were recovered by centrifugation (1,400 g for ten minutes). Cell pellets were solubilized in phosphate-buffered (10 mmol/L potassium phosphate, 150 mmol/L NaCl at pH 7.0) saline with 10 mmol/L EDTA, 1 mmol/L PMSF, 100 μg/mL leupeptin, 100 μg/mL pepstatin A, 0.5% CETAB, and 0.1% Tx-100 on ice for 30 minutes. The solubilized cells were clarified by ultracentrifugation (100,000 g for 60 minutes in a type 50 Ti Beckman rotor [Beckman Instruments, Fullerton, Calif]) and the supernatant fluid frozen at −80 °C or subjected to analysis immediately.

Endoglycosidase H treatment. Antigen-antibody complexes were released from Cowan cells by adding 50 μL of 0.2% SDS and 5% 2-mercaptoethanol and heating the suspension in a boiling water bath for two minutes. Free Cowan cells were removed by centrifugation (12,000 g for one minute) and the supernatant diluted to 500 μL with 150 mmol/L citrate buffer (pH 5.5) to reduce the concentration of SDS to .02%. Five microliters of 100 mmol/L PMSF was added to inhibit proteolysis during the deglycosylation. A quantity of 50 ImU of endoglycosidase H (endo H) was added and the sample incubated at 37 °C for 24 hours with constant agitation. As control, 50 μL of citrate buffer was added to an identical sample. At the end of the incubation period, 50 μL of 100% trichloroacetic acid (TCA) was added and the sample placed on ice for 30 minutes. The resultant precipitate was washed twice in 500 μL of 50% methanol and then dissolved in 100 μL of gel sample buffer, heated in a boiling water bath for two minutes, and electrophoresed as will be described.

RESULTS

Use of HL-60 cell line. Because PMNs isolated from venous blood synthesize little or no protein,[25] it was necessary to use the HL-60 cell line to analyze MPO biosynthesis. To verify that HL-60 cells were appropriate for these studies, the MPO subunits synthesized by HL-60 cells were studied by immunoblotting. MPO purified from human PMNs as previously described,[3] normal PMNs, and HL-60 cells were electrophoresed into a 10% polyacrylamide gel under reducing conditions, followed by electroblotting of separated proteins to nitrocellulose paper. Autoradiography of the resulting immunoblot (Fig 1) demonstrated that purified MPO (lane 1), HL-60 cells (lane 2), and normal PMNs (lane 3) contain the same MPO subunit peptides of 59 kDa (α subunit) and 13.5 kDa (β subunit). In addition, the anti-MPO antiserum also identified an 89-kDa peptide present in HL-60 cells and normal PMNs. When the antiserum was adsorbed with purified MPO prior to treatment of the nitrocellulose paper, this 89-kDa peptide as well as the normal subunits of MPO were no longer detected (data not shown). These observations indicate that the 89-kDa species is immunochemically related to normal MPO and that HL-60 cells provide a suitable model to study MPO biosynthesis.

In vitro translation. To identify the primary translation product of MPO mRNA, RNA isolated from HL-60 cells was translated in a micrococcal nuclease-treated rabbit reticulocyte lysate system and the products immunoprecipitated. The primary translation product was an 80-kDa peptide (Fig 2) that represented 0.16% of the total TCA-precipitable counts when translation was performed for 60 minutes. The addition of 3% polyethylene glycol or 50% $(NH_4)_2SO_4$ to the supernatant remaining after the immunoprecipitation failed to recover additional MPO-related peptides. This finding confirms recent reports of the primary translation product of MPO mRNA from HL-60 cells.[40-42] This result demonstrates that the nonglycosylated peptide backbone of the precursor of MPO has a molecular weight of

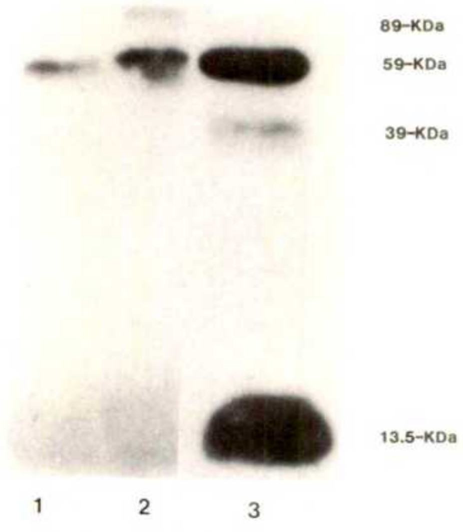

Fig 1. Immunoautoradiographic analysis of MPO peptides. Purified MPO from human PMN (lane 1), undifferentiated HL-60 cells (lane 2), and normal PMNs (lane 3) were electrophoresed into a 13% acrylamide gel and the separated proteins transferred electrophoretically to nitrocellulose paper and the subsequent immunoblot exposed to ^{125}I-protein A. Anti-MPO serum identified predominantly the α (59 kDa) and β (13.5 kDa) subunits of MPO in all three samples. In HL-60 cells and normal PMNs an 89-kDa peptide was evident that was absent from purified MPO.

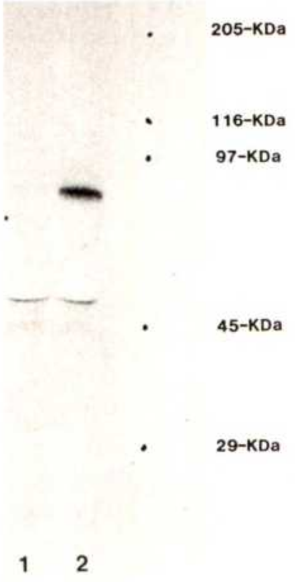

Fig 2. Synthesis of MPO in vitro. A rabbit reticulocyte lysate was supplemented with amino acids (except methionine) and treated with micrococcal nuclease to inhibit translation of endogenous RNA. The lysate was primed with no RNA (lane 1) or 15 μg of RNA isolated from HL-60 cells (lane 2) and proteins labeled with [^{35}S]-methionine. Products were immunoprecipitated with anti-MPO and subjected to SDS-PAGE followed by autoradiography of the dried gel. The primary translation product for MPO synthesis was a single peptide that migrated with a molecular weight of 80 kDa under reducing conditions. Residual activity of endogenous RNA in the reticulocyte lysate accounted for the other labeled proteins seen (lane 1).

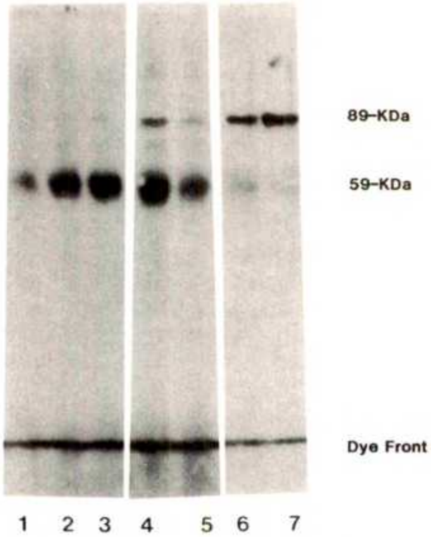

Fig 3. Immunoautoradiographic analysis of MPO peptides in subcellular fractions separated on a gradient of Percoll. Percoll was removed from fractions collected from the gradient prior to separation by SDS-PAGE in a 10% polyacrylamide gel and immunoblotting. Samples from fractions 17 to 19 were electrophoresed in lanes 1 to 3. These fractions contained one of the two major peaks of peroxidase activity and in the immunoblot contained the 59-kDa subunit of MPO. Fractions 22 and 23 were electrophoresed in lanes 4 and 5. These fractions contained the remaining peroxidase activity in the gradient as well as galactosyl transferase and alkaline phosphatase and had both 89-kDa and 59-kDa peptides identified by the immunoblot. Fractions 25 and 26 were the least dense fractions in the gradient and had no peroxidase activity but peak activities for galactosyl transferase and alkaline phosphatase. The immunoblot of these fractions, lanes 6 and 7, contained predominantly the 89-kDa peptide. The 13.5-kDa species migrated with the dye front in a 10% gel.

sufficient size (80 kDa) to account for the α and β subunits (59 + 13.5 kDa) covalently linked into a single polypeptide. After HL-60 cells were induced to differentiate, either by culturing for three days in conditioned medium containing γ-interferon or by culturing for five days in 1.3% dimethyl sulfoxide, their isolated RNA failed to translate for any MPO-related material in vitro (data not shown). Of note, these induced cells still produced functional RNA since the total TCA-precipitable counts from translations primed with RNA from induced cells was higher than control lysates with no RNA added. Thus, this suggests that under the induction conditions used, differentiation was associated with cessation of transcription of the gene for MPO, indicated by the absence of MPO synthesis.

Analysis of subcellular fractions. HL-60 cells were disrupted by nitrogen cavitation and the subcellular organelles separated on a density gradient of Percoll. The immunoblots of individual fractions are shown in Fig 3 and demonstrate that the 89-kDa peptide and the normal subunits of MPO sedimented into separate subcellular fractions. Samples from fractions 17 to 19 from the gradient were electrophoresed in lanes 1 to 3 of the immunoblot shown in Fig 3 and comigrated with the major peak of peroxidase activity. The only MPO peptide detected in this fraction was the 59-kDa α subunit, whereas the β subunit ran at the dye front in the 10% acrylamide gel. Fractions 22 and 23, electrophoresed in lanes 4 and 5 of the immunoblot, contained both the α subunit and the 89-kDa peptide. These fractions contained a small amount of peroxidase activity and comigrated with activity of galactosyl transferase, a marker for *trans*-Golgi. Immunoblots of fractions 25 and 26 contained only the 89-kDa peptide (lanes 6 and 7) and cosedimented with peak activities of galactosyl transferase and alkaline phosphatase, a marker of plasma membrane.

Further work on improving resolution of species in the less dense regions of the gradient is underway,[43] and the identity of subcellular organelles containing 59-kDa and 89-kDa species cannot be absolutely ascertained from this analysis. It is clear from these initial studies that (1) the 89-kDa peptide and mature MPO were in separate subcellular compartments; (2) the 89-kDa peptide was in fractions from the gradient that were less dense than those containing lysosomes and cosedimented with biochemical markers for Golgi and plasma membranes; and (3) the 89-kDa peptide has no peroxidase activity, whereas fractions with the 59-kDa α subunit also had peroxidase activity.

Pulse labeling experiments. Uptake of [^{35}S]-methionine by HL-60 cells starved in a methionine-free medium prior to labeling was rapid, and immunoprecipitable material was detectable after five minutes of labeling (data not shown). In pulse-chase experiments cells were labeled for 30 minutes because these conditions resulted in the greatest number of counts incorporated into an MPO-related peptide without processing of the initial product to more mature species. The earliest labeled peptide immunoprecipitated was an 89-kDa peptide (Fig 4, lane 1).

Pulse-chase experiments demonstrated that this 89-kDa was very slowly processed into normal MPO subunits (Fig 4). Thirty minutes after the addition of cold methionine, a fraction of the 89-kDa species was processed to a 47-kDa peptide intermediate (data not shown) that was further processed to a 39-kDa peptide, the molecular weight of the α subunit of MPO when purified MPO is electrophoresed under nonreducing conditions.[3,11] After five hours of chase the major peptides immunoprecipitated had molecular weights of 89,000, 39,000, and 47,000 (lane 2). Subsequently, the α and β subunits of native MPO were seen after 20 hours of chase, migrating at 59,000 and 13,500 (lane 3). Of note, the predominant species immunoprecipitated were 89-kDa and 39-kDa peptides after a prolonged period of chase (100 hours, lane 5). Samples of immunoprecipitated supernatant contained small amounts of the 89-kDa peptide (data not shown).

Endoglycosidase treatment. Endo H is an enzyme that digests glycoproteins with high-mannose side chains at the proximal *N*-acetylglucosamine-*N*-acetylglucosamine linkage.[44] To characterize the glycosylated intermediates of MPO biosynthesis, immunoprecipitates of extracts from cells pulsed for 30 minutes were treated with endo H. The autoradiography of immunoprecipitated protein incubated in the presence (lane 2) of endo H is shown in Fig 5. The 89-kDa species migrated as a peptide of 79 kDa after treatment with endo H, indicating that the 89-kDa peptide contains high-mannose side chains. In addition, treatment with endo H reduced the 59-kDa subunit to 53 kDa, whereas the 39-kDa species was essentially unchanged. The 39-kDa peptide migrates as a broad band even after digestion with endo H, suggesting that the oligosaccharide side chains on this peptide are heterogeneous and only a fraction are of the high-mannose type. When the autoradiograph is developed sufficiently long to visualize well the 13.5-kDa subunit (not shown), it appeared unaltered by endo H digestion. These observations suggest that the precursor to MPO is glycosylated in the Golgi by the addition of high-mannose side chains to the nascent peptide. In addition, some of the high-mannose side chains are not processed in the Golgi and are present in the α subunit of mature MPO.

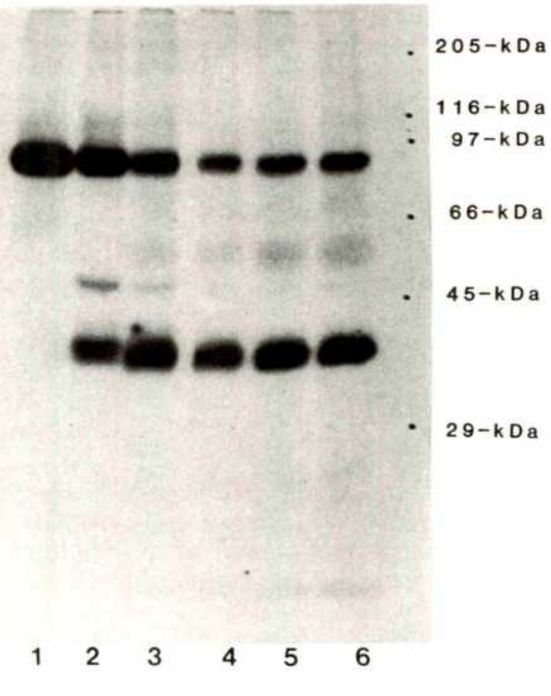

Fig 4. Synthesis of MPO by HL-60 cells. HL-60 cells were starved in methionine-free RPMI 1640 with 10% dialyzed FCS for 30 minutes prior to the addition of [^{35}S]-methionine (100 μCi/mL). Cells were labeled for 30 minutes, after which time a sample was taken (zero chase, lane 1) and cold methionine (final concentration, 1 mmol/L) was added. Samples were taken at 5, 20, 44, 68, and 100 hours (lanes 2 to 6 respectively), immunoprecipitated, reduced, and electrophoresed into a gradient gel (8% to 14% acrylamide and 0% to 14% glycerol). Initially, an 89-kDa peptide was labeled, and it was processed to a 47-kDa intermediate and a 39-kDa peptide that was apparent at five hours of chase (lane 2). After 20 hours of chase (lane 3), the α (59 kDa) and β (13.5 kDa) subunits of mature MPO were identified with a gradual decrease in the 89-kDa peptide.

DISCUSSION

Since first identified as verdoperoxidase by Agner in 1941,[45] MPO has been studied extensively, and its important role in the microbicidal activity of PMNs via the MPO-H_2O_2-halide system has been firmly established.[46] Despite extensive investigation of the structure[2] and function[46] of

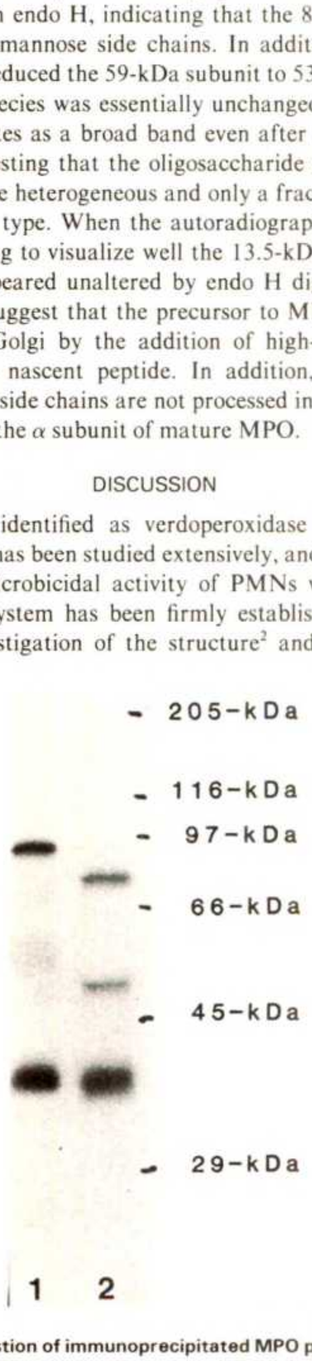

Fig 5. Digestion of immunoprecipitated MPO peptides by endo H. Labeled HL-60 cells that had been chased for 68 hours were immunoprecipitated. The immunoprecipitates were incubated at 37 °C for 24 hours without (lane 1) or with (lane 2) 50 mIU of endo H. Endo H digested the 89-kDa peptide to 79 kDa, the 59-kDa peptide to 53 kDa, and the 39-kDa peptide to 37 kDa, indicating that these glycoproteins contain high-mannose side chains. After more prolonged exposure (not shown), this autoradiograph demonstrated that the 13.5-kDa subunit was resistant to endo H digestion.

MPO, studies of MPO biosynthesis are few in number,[40,41,47] in part reflecting the terminally differentiated state of the PMN characterized by little, if any, protein synthesis.[25] Currently there are at least three general reasons for an interest in MPO biosynthesis.

First, there have been significant advances in understanding cotranslational and posttranslational events during protein synthesis by various types of cells.[48-54] Of relevance to MPO biosynthesis are studies demonstrating the importance of posttranslational modification and phosphorylation of oligosaccharide side chains for the proper transport of lysosomal enzymes from the endoplasmic reticulum to the lysosome.[48-50] Many of the lysosomal enzymes studied to date follow a similar pattern of production and processing. The primary translation product has an amino-terminal sequence that promotes insertion of the nascent peptide through the membrane and into the lumen of the endoplasmic reticulum. As cotranslational events, the insertion sequence is removed by a signal peptidase, and the nascent peptide is glycosylated at the amide nitrogen of certain asparagines in the peptide (*N*-linked glycosylation). Glycosylation is initiated by transfer en bloc of a high-mannose oligosaccharide chain (Glc_3 Man_9 $GlcNAc_2$) from the lipid carrier dolichol to the asparagine. The oligosaccharide side chains are subsequently modified during passage through the Golgi where mannose-6-phosphate residues are incorporated into some of the side chains. These phosphomannosyl groups allow the glycoprotein to bind to the mannose-6-phosphate receptor on the lysosomal membrane and thereby translocate to the lysosome. Hasilek et al[42] have recently demonstrated ^{32}P incorporation by the α subunit during MPO synthesis but were unable to demonstrate mannose-6-phosphate receptors. Newly synthesized glycosylated precursors of lysosomal enzymes, so-called pro forms, are generally larger than the corresponding mature enzyme, and proteolytic conversion to the mature form appears to be closely linked to transport to the lysosome.[39,51]

The second reason to examine MPO biosynthesis is that production of MPO is closely linked to myeloid differentiation.[40,41] Two recent studies have demonstrated that MPO synthesis by promyelocytic HL-60 cells ceases when the cells are chemically induced to differentiate to more mature cells, either macrophages or granulocytes.[40,41] The induced cells had no MPO-specific mRNA as assessed in an in vitro translation system, although functional RNA was still transcribed by induced cells. This suggests that cessation of transcription of the MPO gene is responsible for the termination of MPO synthesis seen during myeloid differentiation. These studies of MPO biosynthesis may provide information on the control mechanisms for protein synthesis during myeloid differentiation and insight into events associated with abnormal differentiation in neoplasia.

The third reason to study MPO biosynthesis is to examine the genetic basis for MPO deficiency, a common hereditary disorder of PMNs.[12] In a previous study,[3] sensitive immunoblot analysis of peroxidase-deficient PMNs separated by PAGE demonstrated that MPO-deficient PMNs lack the normal α and β chains of MPO but contain an MPO-related 89-kDa peptide that is also present in normal PMNs. The presence of this 89-kDa peptide is consistent with the hypothesis that MPO deficiency reflects a failure of developing myeloid cells to compartmentalize MPO into azurophilic granules, perhaps secondary to defective posttranslational processing of the enzyme precursor.

Studies presented here suggest that biosynthesis of MPO likely follows the same general pattern previously outlined for other lysosomal enzymes. The nonglycosylated primary translation product synthesized in vitro had a molecular weight of 80,000 and is thus a peptide of sufficient size to accommodate the α and β chains of mature MPO (59 kDa + 13.5 kDa) linked covalently into a single precursor. The earliest form of MPO synthesized in vitro in pulse experiments was an 89-kDa protein identified within five minutes of labeling. Treatment of the 89-kDa peptide with endo H, an enzyme that cleaves between proximal *N*-acetylglucosamine groups on high-mannose side chains, resulted in a 79-kDa peptide, demonstrating the presence of high-mannose side chains on the 89-kDa peptide. In preliminary experiments (data not shown), HL-60 cells labeled in the presence of tunicamycin, an antibiotic that prevents the initial transfer of the high-mannose oligosaccharide from dolichol to the nascent peptide backbone,[55] produced a core peptide of 75 to 77 kDa. This core glycosylation occurred very early in biosynthesis and was very likely a cotranslational event whereby the signal peptide was removed from the 80-kDa primary translation product and the core peptide of approximately 77 kDa glycosylated at selected asparagines to produce the 89-kDa peptide, although there is no direct evidence for a signal peptide presented here. The 89-kDa species was identified in immunoblot analysis of subcellular fractions of HL-60 cells separated in a discontinuous gradient of Percoll. This peptide was localized in a subcellular organelle(s) that was less dense than lysosomes and that cosedimented with enzyme markers for Golgi and plasma membrane.

When labeled HL-60 cells were chased with a 1,000-fold excess of unlabeled methionine, the 89-kDa peptide was slowly processed, first to an intermediate 47-kDa peptide that was converted to a 39-kDa peptide beginning within two hours of chase (data not shown). After five hours of chase, the α and β subunits of mature MPO were immunoprecipitated, although the 39-kDa peptide remained prominent late into the chase phase. The 89 kDa was processed very slowly and was still present after 100 hours of chase. The relatively slow rate of processing of the MPO precursor has been noted by Hasilek et al who recently reported persistence of the precursor glycoprotein after as late as 70 hours of chase.[42]

In preliminary experiments, SV8 protease digestion of the 89-kDa species generated seven peptides of mol wt <49,000, as did digestion under identical conditions of the immunoprecipitation after 24 hours of chase (data not shown). The persistence of the 89-kDa peptide late into the chase makes it impossible to compare a proteolytic digest of isolated 89 kDa with that of isolated 59-, 39-, and 13.5-kDa subunits. Kinetics of labeling in the pulse-chase experiments coupled with these findings are sufficient evidence for a product-precursor relationship between the 89-kDa peptide and mature MPO. In fact, few examples exist in the literature of such a relationship substantiated by peptide or sequence analysis of the precursor and the product,[56] although studies are currently underway toward that end.

Endo H treatment modified the 89-kDa glycoprotein to a 79-kDa peptide regardless of the time during the chase at which the 89-kDa material was immunoprecipitated, indicating persistence of the high-mannose side chains on the 89-kDa peptide. Digestion of the processed peptides with endo H demonstrated that the α subunit has susceptible high-mannose side chains and was deglycosylated to a 53-kDa peptide. In contrast, the β subunit appeared unaffected by endo H treatment. These findings extend a recent study by Olsen and Little[11] in which they reported the results of deglycosylation of the purified subunits of MPO using trifluoromethanesulphonic acid. With this treatment the α subunit (57 kDa in their study) was converted to 52 kDa, whereas the β subunit was unchanged. Therefore not only are the carbohydrate side chains in MPO limited to the α chain but they are primarily high-mannose oligosaccharides.

Purified MPO has subunits of 59 kDa and 13.5 kDa in a 1:1 ratio,[3-8] but under nonreducing conditions has subunits of 59 kDa, 39 kDa, and 13.5 kDa in a 1:1:2 ratio.[9] The 39-kDa species has been observed by numerous investigators studying MPO structure,[3,4,5,11,47,57] but there is no consensus as to its relationship to the α and β chains of MPO. Investigators have attributed its presence to anomalous migration of unreduced 59-kDa peptide in polyacrylamide gels,[57] boiling samples in SDS prior to electrophoresis,[47] reducing samples for electrophoresis during or after heat treatment,[11] proteolytic digestion of the 59-kDa peptide,[4] and contamination of the MPO. Two-dimensional maps of chymotryptic digests of iodinated 59-kDa and 39-kDa species separated by PAGE are similar but not identical[9] and clearly demonstrate that these species are very closely related. Data presented here demonstrate the differences in the carbohydrate composition of the 59-kDa and 39-kDa species, the latter being relatively insensitive to endo H. Although the interrelationship between the 59-kDa and 39-kDa species is still the subject of ongoing work, the 39-kDa peptide was the predominant species during pulse-chase experiments.

The data presented here provide a foundation for further examination of MPO synthesis, processing, and packaging in normal myeloid cells. The findings presented are consistent with the hypothesis that the genetic defect underlying MPO deficiency is different from that previously proposed.[13-18] The presence of the 89-kDa MPO-related glycoprotein in MPO-deficient PMNs suggests that the deficient cells express the gene for MPO synthesis but that some event in the cotranslational/posttranslational processing is defective. This defect results in production of an aberrant form of MPO that, because of abnormal processing, is not packaged in the azurophilic granules during the promyelocytic stage of development. Small amounts of pro-MPO remain in the vestigial Golgi of peripheral blood PMNs where it appears as the faint band at 89 kDa when normal and MPO-deficient PMNs are analyzed by immunoblotting.[3] MPO-deficient cells, which might be expected to contain larger amounts of pro-MPO as a reflection of faulty processing, may behave in a fashion akin to fibroblasts in I cell disease.[58] The latter lack *N*-acetylglucosamine phosphotransferase and therefore are unable to produce the recognition marker mannose-6-phosphate for transport of lysosomal enzymes. In the absence of the necessary targeting structures to direct and transport the proteins correctly to the lysosome, the aberrant form of the lysosomal enzyme is released from the cell into the medium rather than properly stored in the lysosome. In an analogous manner, promyelocytes of MPO-deficient individuals may secrete relatively large amounts of pro-MPO into their immediate environment in the bone marrow. Work is in process to test this hypothesis in order to identify the genetic defect in hereditary MPO deficiency.

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Isolation of Circumferential Microtubules From Platelets Without Simultaneous Fixation

By James G. White, E. Radha, and Marlys Krumwiede

Circumferential bands of microtubules (MT) support the discoid shape of resting platelets and participate with the contractile apparatus in shape change and internal contraction following activation. Elucidation of interactions between the circumferential coils and proteins of the stable and contractile cytoskeleton is essential for understanding MT function in platelet physiology. A previous investigation demonstrated that the circumferential rings can be isolated intact from resting platelets following simultaneous exposure to glutaraldehyde and Triton X-100. However, the use of fixation prevented the characterization of protein interactions. The present study has circumvented this problem by developing a procedure for isolating intact microtubule coils from detergent-treated platelets without the use of fixative agents. Incubation of the platelets for intervals of 30 to 60 minutes with the microtubule-stabilizing agent taxol preserved the circumferential bundle after extraction with Triton X-100 even after washing five times. The procedure has made it possible to carry out protein studies on isolated microtubule rings and associated proteins.

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THE CIRCUMFERENTIAL microtubule (MT) is an important constituent of the platelet cytoskeleton. It supports the discoid shape of resting platelets[1-3] and participates in the process of internal contraction when the cells are stimulated.[4,5] The precise mechanisms through which circumferential MT exert these functions, however, are unclear.[6]

To evaluate the role of the circumferential MT in platelet physiology, we have recently developed a procedure for isolating intact MT coils from platelets in suspension.[7] The method has been useful for studying the fate of MT in activated platelets,[8] but biochemical and physiologic experiments have been limited because glutaraldehyde was needed in the preparative procedure. In recent investigations we have found a way to avoid the presence of a fixative during the separation of MT coils from suspended platelets. The addition of a relatively large concentration of taxol,[9] an MT-stabilizing agent, obviated the need for glutaraldehyde and permitted the recovery of large numbers of MT coils in a nearly native state.

MATERIALS AND METHODS

General. After informed consent, blood was obtained from healthy adult donors whose platelets had been evaluated in many previous studies from our laboratory.[4,10,11] Samples obtained by venipuncture were mixed immediately with citrate-citric acid, pH 6.5 (93 mmol/L sodium citrate, 70 mmol/L citric acid, and 140 mmol/L dextrose), in a ratio of nine parts blood to one part anticoagulant. Platelet-rich plasma (PRP) was separated from whole blood by centrifugation at 100 g for 20 minutes at 23 °C and maintained at room temperature until used in specific experiments.

Concentration of platelets. Adenosine (5 mmol/L) and theophylline (3 mmol/L) were added to the citrate anticoagulant described previously to inhibit platelet activation.[12] Five milliliters of PRP were combined with an equal volume of the citrate anticoagulant and centrifuged at 1,500 g for 30 minutes to obtain pellets. The supernatant was decanted and the platelets gently resuspended in 1 to 2 mL of PRP. Concentrated samples were maintained in suspension at room temperature. The procedure resulted in a three- to four-fold concentration of platelets, and a substantial proportion of the cells were discoid after resuspension in PRP.

Samples of concentrated PRP, 0.5 mL in volume, were combined with taxol,[9,13,14] an MT-stabilizing agent, at a final concentration of 2×10^{-4} mol/L. Taxol was obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md. The agent was dissolved in dimethyl sulfoxide at a concentration of 10 mmol/L. The platelets were incubated with taxol for one hour before lysis.

Platelet lysis. A buffer designed by Small[15] to preserve cytoskeletal elements (NaC1, 127 mmol/L; KCl, 5 mmol/L; Na_2HPO_4, 1.1 mmol/L; KH_2PO_4, 0.4 mmol/L; $NaHCO_3$, 4 mmol/L; glucose, 5.5 mmol/L; $MgCl_2$, 2 mmol/L; ethylene glycol tetra-acetic acid, 2 mmol/L; piperazine-N,N^1-Gis (2-ethanesulfonic acid) (PIPES), 5 mmol/L; pH 6.0 to 6.1) was combined with Triton X-100 (Sigma, St Louis) at a final concentration of 10%. A 100-μL volume of this solution was combined with 0.5 mL of taxol-treated, concentrated platelets. The detergent caused almost immediate clearing of the cloudy cell suspension.

Preparation of whole mounts. Single drops of the extracted platelets were placed on formvar-coated, carbon-stabilized grids for 2½ minutes, rinsed with cytoskeleton buffer, and examined unfixed or after postfixation for two or more hours in 2.5% glutaraldehyde in the same buffer.[16,17] Grids were then rinsed twice with distilled water and drained briefly on the edge with filter paper. Finally, the grids were passed through four drops of 3% sodium silicotungstate, drained of excess stain, and allowed to air dry.[15]

Preparation for scanning electron microscopy. Large drops of the supernatant from extracted platelets were mounted on circular glass disks. In addition, the lysate and samples of washed, resuspended MT were deposited on millipore filters. After contact with surfaces for 2½ minutes, they were rinsed with cytoskeletal buffer and postfixed in 2.5% glutaraldehyde as previously described for whole mount preparations. The coils were then dehydrated in a graded series of alcohols, substituted with freon, and dried by the critical point method of Anderson in a Bomar apparatus (Bomar Instruments, Tacoma, Wash).[18] After coating with a 50 Å layer of carbon and gold, the coils were examined in a Cambridge S4D-10 scanning electron microscope (Cambridge Instruments, Cambridge, England).

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Preparation for polyacrylamide gel electrophoresis (PAGE). Six to eight detergent-extracted, 0.5-mL platelet samples were combined and centrifuged to obtain a pellet. Clear supernatant was discarded and the pellet resuspended in Hanks' balanced salt solution (HBSS) containing taxol at 2×10^{-4} mol/L. The washing procedure was repeated five times and the final pellet resuspended in 0.5 mL of HBSS with taxol. The protein content of the platelet concentrate and isolated MT coils was measured by the method of Lowry et al.[19] Sodium dodecyl sulfate (SDS)-PAGE was performed according to the method of Laemmli.[20] A gradient slab of 8% to 15% was used for analyzing the protein profile.[21] The samples were dissolved in an SDS sample buffer and boiled for two minutes. Protein standards were electrophoresed for molecular weight estimations. Gels were stained with brilliant Coomassie blue R and scanned with a Kratos spectrodensitometer (Kratos Analytical Instruments, Westwood, NJ) connected to a Hewlett-Packard integrator (Hewlett-Packard, Palo Alto, Calif).

Immunoblot preparation. PAGE gels of the isolated MT coils prepared as previously described were transferred to nitrocellulose electrophoretically according to the method of Towlin et al.[22] After blotting, the nitrocellulose was incubated with gelatin to block nonspecific protein-binding sites. The blot was then incubated with a monoclonal antibody to β-tubulin of mouse origin (Cooper Biomedical, Malvern, Pa). The immune complex was detected with peroxidase-labeled antibody to mouse IgG after incubation in 4-chloro-1-napthol.

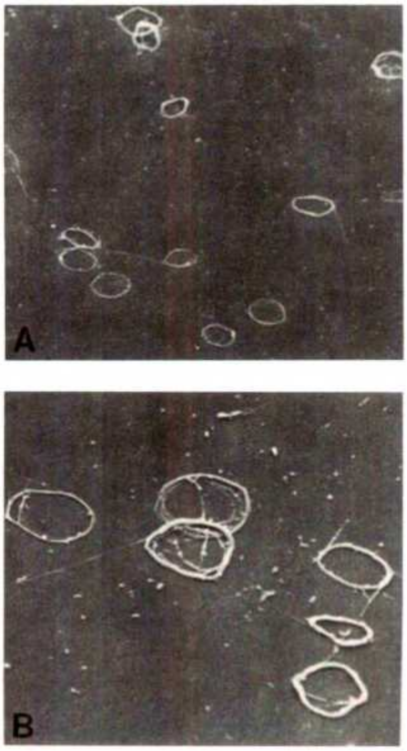

Fig 1. Low- and high-magnification scanning electron micrographs of isolated marginal band MT. The MT coils have retained their concentric organization throughout the isolation procedure and multiple washings and appear randomly dispersed on the surface of the disks. Membranes and cytoplasmic constituents are virtually absent from the coiled MT. Original magnifications (A) ×2,600; (B) ×6,500; current magnifications (A) ×1690; (B) ×4225.

RESULTS

Morphology. MT coils recovered by detergent lysis from platelets incubated for an hour with a high concentration of taxol were essentially identical in appearance to the coils isolated from platelets after simultaneous exposure to detergent and glutaraldehyde fixative. Examination of glass disks (Figs 1A and 1B) and millipore filters (Figs 2A and 2B) in the scanning electron microscope revealed fields that were virtually free of any other structures but MT coils. Whole-mount preparations viewed in the transmission microscope were also nearly devoid of cellular debris. MT coils consisted of several concentric rings, ranging from three to 12 with a median of eight. Two free ends were observed extending from some coils, but multiple free ends were also common (Figs 3A and 3B). MT coils were more loosely arranged in the absence of glutaraldehyde fixation during isolation or mounting, but the basic circular configuration of the loops was well maintained in most examples.

Biochemistry. Depending on the amount of protein applied to the gel, SDS-PAGE of proteins solubilized from multiply washed MT rings revealed a dense single or double band migrating with the same mobility as the tubulin standard (Fig 4). In addition to tubulin, several other proteins were evident on the gels. A band at approximately 210 K may represent an MT-associated protein (MAP). Two or three additional protein bands with apparent molecular

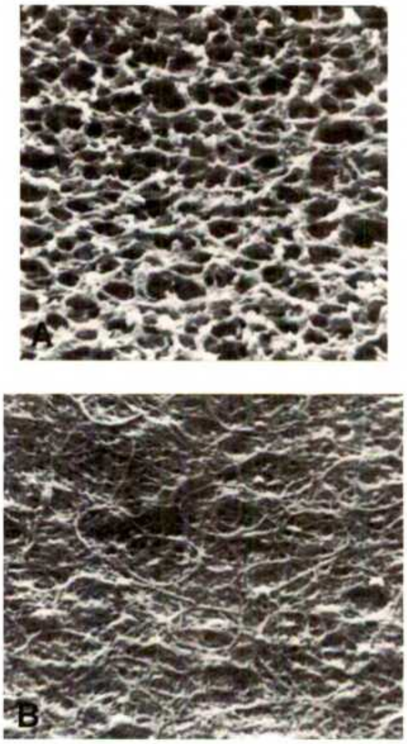

Fig 2. Scanning electron photomicrographs of nucleopore filters. (A) The filter has not been exposed to MT. (B) The surface has been exposed to the multiply washed and resuspended MT obtained from eight samples of detergent treated platelet concentrate, each 0.5 mL in volume. The surface is buried in coils. Original magnifications ×6,000; current magnifications ×3900.

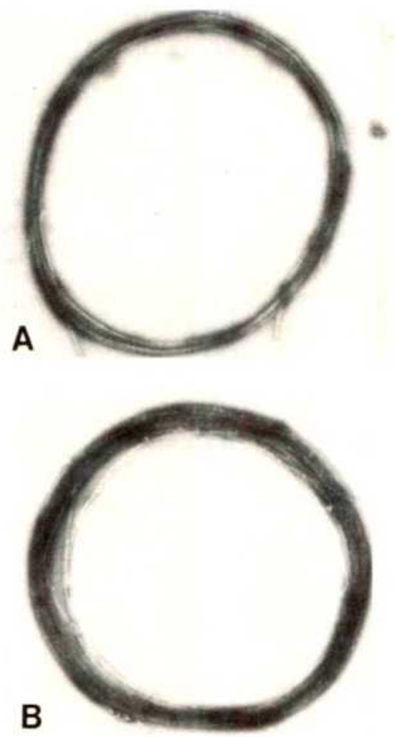

Fig 3. Negative-stain whole mounts of MT coils prepared without glutaraldehyde fixation from detergent-treated platelets after repeated washing and resuspension. The tightly coiled appearance is well preserved in the two examples. More than two loose ends are visible on each coil, but the increase may be due to fracture during preparation. Original magnifications ×18,000; current magnifications ×11,700.

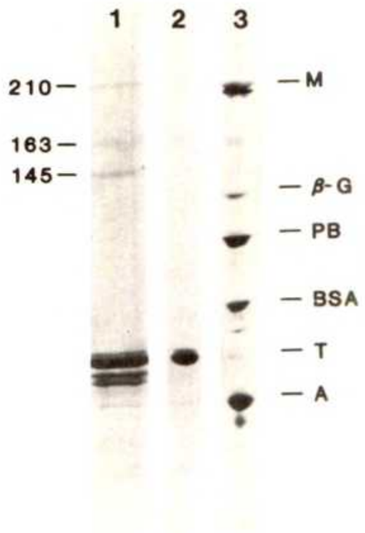

Fig 4 SDS-PAGE (5% to 15% gradient) separation of proteins solubilized from isolated MT coil preparation after five washings and resuspensions in HBSS (channel 1). The gels were stained with brilliant Coomassie blue R. Channel 2 contains partially purified brain tubulin (T), and channel 3 contains several standard proteins for molecular weight estimation, including myosin (M), β galactosidase (3G), phosphorylase B (PB), bovine serum albumin (BSA) and actin (A). α- and β-tubulin are not well separated in the standard or experimental samples because of the high protein concentration. A protein band at approximately 210,000 mol wt on the experimental gel may represent an MT associated protein like MAP.[2] The nature of the other HMW proteins is not known. The actin band is usually more prominent in the experimental gels. Several protein bands between actin and tubulin remain with isolated coils throughout isolation and washing procedures. They may represent LMW MAPs.

weights above 100,000 may represent breakdown products of high–molecular weight (HMW) proteins since proteolytic inhibitors were not included during lysis or washing. A low–molecular weight (LMW) band with the mobility of the actin standard was constantly present. Between tubulin and actin were two or three bands that may represent LMW MAPs. Immunoblots prepared from the isolated MT coils, incubated with a monoclonal antibody to tubulin, and stained with a peroxidase labeled second antibody revealed a specific reaction with platelet tubulin (Fig 5).

Densitometry of PAGE gels revealed that tubulin constituted 43% to 48% of the protein on the gels. Measurement of the protein content of the concentrated platelet suspension and multiply washed MT coils, adjusted on the basis of the densitometric observations, revealed that tubulin constituted about 3.3% to 3.8% of the total platelet protein.

DISCUSSION

The present report has described a relatively simple way to stabilize the circumferential MT supporting the discoid shape of resting platelets during isolation and purification. Taxol, an MT-stabilizing agent,[9] was added to platelet suspensions at twice the concentration used in previous studies.[13,14] Platelets were incubated for an hour and then lysed in a cytoskeletal buffer containing Triton X-100. The presence of taxol prevented disassembly and disorganization of MT coils during rupture of the cell membranes by detergent. Examination of glass disks prepared for scanning electron microscopy after contact with drops of the lysed platelet suspension for 2½ minutes revealed MT coils evenly distributed on the surface. Millipore filters on which MT coils isolated from 4 mL of cleared suspension had been deposited were completely covered by coils. Thus, the procedure appears to provide a simple way to obtain susbstantial numbers of MT coils for evaluation without requiring the use of glutaraldehyde or other fixatives.[7]

The morphology of unfixed MT coils was generally well preserved by the pretreatment with taxol. The loops making up the MT coils had separated in some examples, but in most the resemblance to a lariat was well preserved. The number of free ends of MT extending from the coils was variable. Fracture of the delicate structures during attachment to grids and glass coverslips or in the process of air or critical-point drying may have caused some disruption.[7] However, survival of the basic concentric configuration suggests that cohesive forces may be involved in holding loops of the coiled MT together. The nature of these forces is not known but may involve one or several types of MAPs.[23-25]

SDS-PAGE proteins solubilized from the multiply washed MT coils revealed a prominent tubulin band identical in

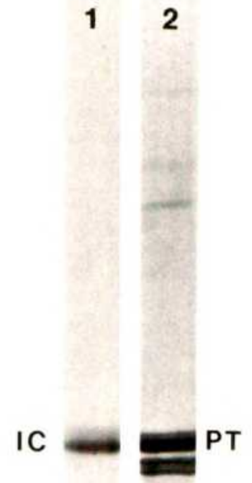

Fig 5. An immunoblot (channel 1) transferred from a PAGE gel of isolated MT coils prepared as described in the text and shown in Fig 4. The blotted proteins were stained with a monoclonal antibody to tubulin followed by a peroxidase-coupled second antibody to the immune complex (IC). Only the protein band (channel 2) identified as platelet tubulin (PT) by its comigration with standard MT protein in the previous Figure is stained by the peroxidase-coupled antibody.

mobility to the brain-derived standard. Immunoblotting and staining with a monoclonal antibody to tubulin confirmed that the band was authentic MT protein. Despite the multiple washings and resuspensions in buffer, several other protein remained associated with MT coils. Both HMW and LMW proteins, including one with a mobility identical to the actin standard, were present in the gels. One of the HMW proteins had a mobility similar to an MAP described previously in platelets and other cell types.[23-25] Two or three protein bands lying between actin and tubulin may also represent MAPs. Studies to characterize these proteins are continuing.

Analysis of the gels by densitometry revealed that tubulin constituted approximately 40% to 50% of the total. Based on protein determinations on the concentrated platelet suspension and the multiply washed coils, the amount of tubulin recovered constituted about 3.3% to 3.8% of the total platelet protein. The recovery obtained in this study agrees well with the figure of 3.1% reported by Steiner and Ikeda.[26]

Taxol has been used previously to facilitate isolation of MT and MAPs from other cell types.[27] However, the procedure involving taxol was used primarily to separate MAPs from MT reassembled after earlier isolation of tubulin from calf brain. The present investigation appears to be the first study to use taxol for maintenance of MT organization observed in the intact cell during isolation and multiple washing. As a result, the proteins remaining in an insoluble state with isolated MT during washing may be very important to MT function in intact platelets. Studies to identify and characterize these proteins by immunoelectron microscopy are in progress.

NOTE ADDED IN PROOF

Following acceptance of this manuscript, a report using a similar approach to isolating the platelet circumferential microtubule has appeared (Kenney DM, Linck RW: The cytoskeleton of unstimulated platelets: Structure and composition of the isolated marginal microtubular band. J Cell Sci 78:1, 1985).

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On the Interaction of Rabbit Antithrombin III With the Luminal Surface of the Normal and Deendothelialized Rabbit Thoracic Aorta In Vitro

By Mark W.C. Hatton, Sue L. Moar, and Mary Richardson

Pure rabbit antithrombin III was isotope labeled (with ^{125}I or ^{3}H) by two different methods; neither procedure caused a loss of antithrombin activity although both methods affected the affinity of the protein for Sepharose-heparin. From segments from freshly excised rabbit aorta, the uptake of isotope-labeled antithrombin III by the endothelium was rapid and saturable, although relatively small compared to the uptake of thrombin; binding of ^{3}H-antithrombin III to the endothelium resembled that of ^{125}I-antithrombin III. Transendothelial passage of antithrombin III into the subendothelial layers (intima-media) was slow and progressive. Endothelium binding was not affected by pretreating the vessel with either heparin, thrombin, or glycosaminoglycan-specific enzymes. Endothelium-bound antithrombin III was not selectively displaced by either heparin or thrombin. In contrast, endothelium-bound thrombin was rapidly dislodged by antithrombin III as a thrombin–antithrombin III complex. The surface of the deendothelialized aorta (ie, subjected to a balloon catheter) bound antithrombin III avidly. Pretreatment of the deendothelialized vessel with glycosaminoglycan-specific enzymes, particularly heparitinase, decreased intima-media binding by up to 80%. ^{125}I-antithrombin III, when bound to the deendothelialized vessel surface, was actively displaced by either heparin, thrombin, or by unlabeled antithrombin III. The relatively poor binding of antithrombin III compared with that of thrombin by the endothelium in vitro supports an earlier proposal (Lollar P, Owen WG: J Clin Invest 66:1222–1230, 1980) that thrombin bound to high-affinity sites, possibly pericellular proteoglycan, of the endothelium is inactivated by plasma antithrombin III in vivo. Such a situation probably holds for large arteries at least.

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IN 1973, Rosenberg and Damus proposed that heparin, a glycosaminoglycan with potent anticoagulant properties, interacted specifically with a plasma protein, antithrombin III, which in turn assumed a state of activation[1]: activated antithrombin III, complexed with heparin, availed itself more readily for reaction with various proteases that arose during hemostatic imbalance to accelerate the anticoagulation process.[2] Recently, Marcum et al[3] have explored in vivo the role of proteoglycans of the vessel wall of the microvasculature[4] in the inhibition of thrombin by antithrombin III. No direct measurements of antithrombin III binding to the endothelium were reported, but speculation was made that such an interaction with pericellular heparinlike proteoglycans takes place.

A study of the binding of, and interaction between, thrombin and antithrombin III to different immobilized glycosaminoglycans showed that, whereas the enzyme bound strongly to either heparin, heparan sulphate, or dermatan sulphate, antithrombin III bound only to heparin.[5] However, numerous analyses of blood vessel glycosaminoglycans have revealed the presence of both heparan sulphate and dermatan sulphate, but not heparin, in the intima-media of arteries.[6] Also, heparan sulphate and dermatan sulphate have both been identified from the extracellular matrix of cultured endothelial cells.[7,8] We proposed[5] that probably thrombin was attracted by pericellular proteoglycan at the endothelial surface and that plasma antithrombin III reacted rapidly with the proteoglycan-bound enzyme. The proposal, which in many ways complemented earlier models[9–11] for the role of heparin in the thrombin–antithrombin III reaction, was supported by a report from Awbrey et al[12] that thrombin had a great affinity for cultured endothelial cells. Later studies showed that thrombin bound strongly to the luminal surface of blood vessels[13,14] and that endothelium-bound thrombin became inactivated rapidly by antithrombin III.[15,16]

Recently, Stern et al[17] have reported that bovine ^{125}I-antithrombin III bound in a specific, time-dependent manner (5.4×10^4 molecules bound/cell at saturation) to bovine aorta endothelium. In contrast, we observed relatively poor binding of rabbit ^{125}I-antithrombin III to rabbit aorta endothelium using a Hautchen technique as a means for direct measurement of endothelium-bound ligand.[18] Furthermore, relatively poor or negligible binding of this protein to cultured endothelial cells from bovine aorta[19] or cornea[20] has been reported. In the following report we have compared the binding of rabbit antithrombin III for the luminal surface of the normal and deendothelialized (ballooned) rabbit thoracic aorta, considered the possible role of pericellular proteoglycan in the binding, and measured some of the properties of endothelium- and subendothelium-bound antithrombin III.

MATERIALS AND METHODS

Materials. The following were obtained from Sigma Chemical Co (St Louis): heparin, dermatan sulphate, nonspecific protease (from Streptomyces griseus), HEPES, protamine, and bovine serum albumin (Cohn Fraction V; BSA). Modified Eagle's medium (MEM) was purchased from GIBCO (Burlington, Ontario); heparitinase, chondroitinases, AC and ABC (all products of Seikagaku Kogyo Co Ltd, Tokyo), and rabbit serum albumin (RSA) were obtained from Miles Inc (Elkhart, Ind); Arvin was from Berk Pharmaceuticals Ltd (Guildford, Surrey, UK), Thrombostat from Parke-Davis Co (Detroit), Enzymobeads from Biorad Ltd (Mississauga, Ontario), S-2238 from Kabivitrum (Stockholm), and Na pentobarbital (Somnotol) from MTC Pharmaceuticals (Hamilton, Ontario).

Na ^{125}I, NaB^{3}H$_4$, and ACS scintillation fluid were purchased from

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Amersham Corp (Oakville, Ontario); NCS tissue solubilizer was from NEN (Lachine, Quebec), and Sepraphore (cellulose-acetate chromatographic strip) was obtained from Gelman Sciences Inc (Ann Arbor, Mich).

Purification of bovine thrombin and rabbit antithrombin III. An ion exchange procedure that was reported originally for isolating thrombin from citrated plasma[21] was modified slightly to purify the enzyme from Thrombostat.[5] The procedures used for determining various properties (ie, purity, activity) of thrombin preparations have been described before.[13,22] The procedure for preparing antithrombin III has been described before.[11,18] Approximately 6.3 mg (±0.5 mg SEM, n = 5) of antithrombin III was obtained from 100 mL of rabbit acid-citrate-dextrose (ACD) plasma using Sepharose-heparin affinity chromatography.[23] Each preparation was assessed for purity by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis.[24] Using loads up to 45 μg, rabbit antithrombin III presented as a single band (mol wt, approximately 63,000) when stained with Coomassie brilliant blue. Immunodiffusion against antiserum to rabbit antithrombin III raised in sheep produced one precipitin line that, after fixation, stained strongly with Coomassie brilliant blue. Lastly, using S-2238 as a substrate for measuring active thrombin,[25] purified antithrombin III (21 pmol/mL of 0.1 mol/L Na phosphate, pH 7.4) caused 60% to 70% inactivation of thrombin (10 pmol/mL) in ten minutes at 37 °C. The addition of heparin (0.5 μg/mL) to this antithrombin III–thrombin mixture produced total inactivation in less than one minute, whereas dermatan sulphate (0.5 μg/mL) did not significantly alter the rate of thrombin inhibition by antithrombin. From these data, antithrombin III was considered to be active as an inhibitor and essentially free from contaminants, including heparin cofactor II. Calculations of protein concentrations were made using $E^{1\%}$ (280) values of 10.5 and 16.2 and molecular weights of 63,000 and 36,000 for antithrombin III and α-thrombin respectively.[1,11]

Radiolabeling of proteins. Iodination, using Enzymobeads, was carried out as suggested by the supplier, Biorad Ltd. Approximately 20 to 50 μg of protein was labeled (30 minutes, 23 °C), centrifuged, and then dialyzed in the presence of 200 μL of 4% RSA-MEM (see the following material) for 16 to 20 hours (4 °C) against MEM (250 mL × 4) as described previously.[22]

Tritiation of antithrombin III was performed using a derivation of the technique of Rice and Means.[26] Approximately 20 to 35 μg (150 μL) of antithrombin III (in 0.3 mol/L Na phosphate, pH 8.0) was added to 10 μL formaldehyde (1.2 μmol) cooled in an ice bath that was placed in a glove box. After standing for one hour, 10 to 20 μL of NaB^3H_4 (1 to 4 μCi) in 0.1 mol/L NaOH was added. Reaction was allowed to progress for ten minutes at 0 °C and then stopped by adding approximately 50 μL glycerol and 0.5 mL 0.3 mol/L Na phosphate (pH 6.0). After one hour at room temperature, the mixture was added to a dialysis bag (diameter, 0.6 cm) containing 200 μL of 4% RSA-MEM (to prevent losses of ^3H–antithrombin III because of adsorption). Dialysis against 250 mL of 0.15 mol/L NaCl and then 250 mL (× 3) of MEM was carried out over 20 hours (4 °C).

All radiolabeled proteins were stored at 4 °C and were used for binding studies for up to four days from the date of labeling. Antithrombin activities of the radiolabeled samples were compared with unlabeled antithrombin III to determine any damaging effects of radiolabeling. The activities were measured by incubating various quantities of a radiolabeled antithrombin III preparation with 8 to 10 IU of α-thrombin for ten minutes at 23 °C; residual thrombin activity was measured by a plasma-clotting assay.[27]

Preparation of defibrinogenated, recalcified rabbit plasma. Blood was collected through a carotid artery cannula into ACD (ratio [vol/vol] of blood to anticoagulant, approximately 9) and centrifuged (9,000 rpm; 30 minutes) to yield ACD plasma. ACD plasma (25 mL) was treated with Arvin (0.1 mL containing 6 IU) for three hours (37 °C). The fibrin clot was removed, and the plasma supernatant was dialyzed against MEM (250 mL × 2) at 4 °C for 20 hours.

Uptake of radiolabeled proteins by the thoracic aorta. New Zealand White male rabbits, weighing between 2.3 and 3.3 kg, were used. The procedures for anesthetizing and heparinizing the animal before slaughter, cannulation (left carotid artery) leading to exsanguination, the excision and flushing of the vessel, and the postexcision handling (ie, defatting of the adventitia) have been described in detail previously.[22] When required, the aorta was deendothelialized after exsanguination by using a Fogarty balloon catheter (Edwards Laboratories Inc, Santa Ana, Calif) as described before.[22]

The glycosaminoglycan-specific enzymes used in these experiments were tested first for contaminating protease activity using ^{125}I-labeled, denatured casein as described before.[28] Only protease-free samples of heparitinase and the chondroitinases were used for aorta treatment. Data on the glycosaminoglycan specificity of each enzyme was supplied by the manufacturer and was not investigated further. Thus, heparitinase was essentially free from heparinase and chondroitinase activities, and chondroitinase AC was free from heparin-, heparan-sulphate–, and dermatan sulphate–degrading activities. Chondroitinase ABC was without activity on heparan sulphate or heparin.

Measurements of the uptake of radiolabeled antithrombin III were performed with 1-cm aorta segments immersed in 1 mL of 4% RSA-MEM, pH 7.4, at 37 °C. For pretreatment experiments, an aorta segment was incubated with a reagent contained usually in 1 mL of 4% RSA-MEM. After treatment, the segment was washed for one minute in MEM (4 mL) before measuring the uptake of the labeled protein. In displacement experiments, after exposure to the labeled protein the segment was rinsed in MEM (4 mL, one minute) and then incubated with the displacing agent, usually in 1 mL 4% RSA-MEM.

After treatment, each vessel segment was separated into three layers: the endothelium by Hautchen preparation (using cellulose acetate strips as described by Pugatch and Saunders[29]), the underlying intima-media, and the adventitia. The latter two layers were separated using cilia forceps. The wall layers were counted for γ- or β-radioactivity as described before.[18,22] Also, radioactivity released from the vessel wall into the medium was counted and analyzed by gel filtration chromatography.

Gel filtration chromatography. To compare the product released from the vessel wall with the initial labeled protein, the displaced radioactivity (in 1 mL 4% RSA-MEM) was immediately frozen and, occasionally, freeze-dried. The reconstituted sample (1-mL vol) was then loaded onto a Sephadex G-200 column (60 cm × 1 cm) equilibrated with 0.01 mol/L Tris-HCl/0.15 mol/L NaCl, ph 7.4; fraction volume was 1.2 mL.

Examination of the aorta wall components after Hautchen preparation. Aorta segments (1-cm length) were opened by cutting along the long axis, and the endothelium was removed on cellulose acetate film. The endothelium, attached to the celluose acetate film, and the remaining vessel wall were fixed in 2.5% glutaraldehyde in 0.1 mol/L Na cacodylate, pH 7.0, and prepared for transmission (TEM) and scanning electron microscopy (SEM) by standard techniques.[30] Cellulose acetate–endothelium preparations were also prepared for light microscopy by staining with 0.07% toluidine blue (10 seconds at 23 °C), rinsing in water, air-drying, and mounting on a glass slide.

RESULTS

Tissue preparation. Preparations of segments of normal (uninjured) aorta incubated in 4% RSA-MEM showed

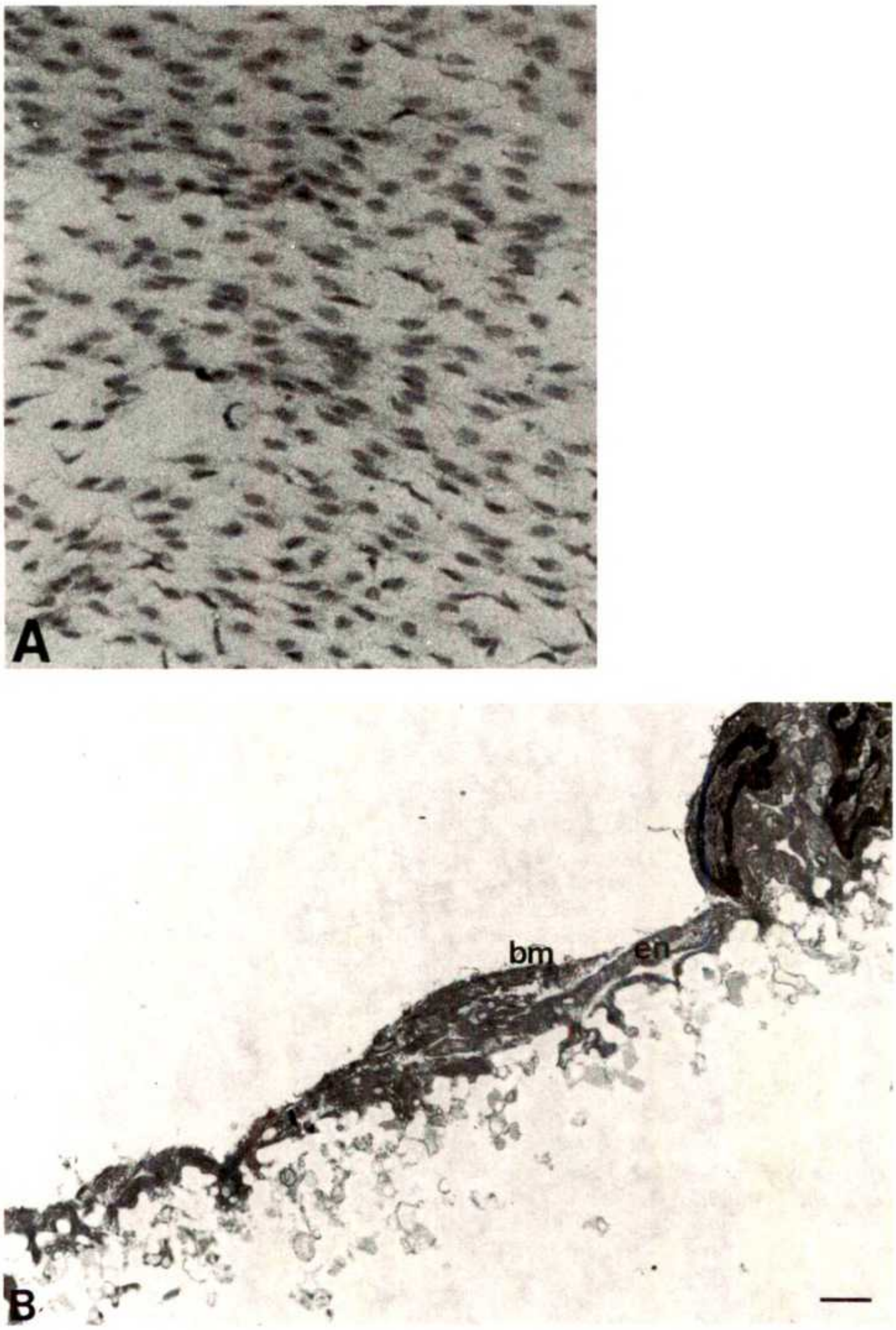

Fig 1. Morphologic examination of rabbit aorta endothelium and subendothelium after exposing the surface to cellulose acetate film. (A) Light micrograph of a toluidine blue–stained Hautchen preparation of endothelium. The endothelial cell layer is complete and indicates that most of the endothelial cell monolayer was removed by this technique (instrumental magnification × 100). (B) TEM of the endothelial cells attached to the cellulose acetate film. Only endothelial cells and remnants of basement membrane have been removed by the cellulose acetate film. Abbreviations: en, endothelial cell; bm, basement membrane; bar, l μ. (C) SEM of the subendothelial connective tissue exposed following cellulose acetate stripping of the endothelium. No remaining endothelial cells are evident. Instrumental magnification × 640 (bar, 100 μ); current magnification × 448.

that the endothelium was almost intact when examined by SEM and the cells showed minimal evidence of injury from examination by TEM. In segments from aortas that had been deendothelialized by balloon catheter the endothelium was completely removed, but remnants of intimal connective tissue remained above the internal elastic lamina. Light microscopy of the endothelium removed by cellulose acetate showed a continuous monolayer of endothelial cells (Fig 1A), and TEM of this preparation confirmed that the endothelial cells were removed with only remnants of the adhering basement membrane (Fig 1B). SEM of the surface exposed after removal of the endothelium confirmed that most of the endothelial cells had been removed, thus exposing the subendothelial connective tissue (Fig 1C).

Effects of radiolabeling on certain properties of antithrombin III. All purified samples of antithrombin III were isolated from plasma not more than six days before labeling. The labeled samples were rechromatographed on Sepharose-heparin to determine whether the labeling process had damaged the heparin-binding function of the protein (Fig 2).

Unlabeled antithrombin III was distributed into approximately 20% to 30% of the protein that was unbound by heparin and 70% to 80% that during gradient elution

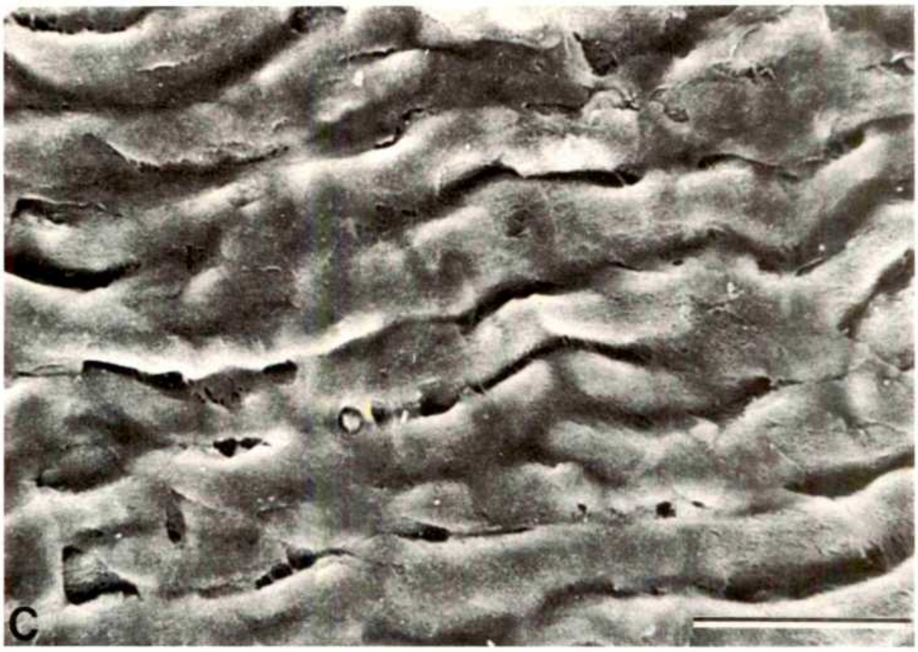

Fig 1. (Cont'd)

emerged as a sharp peak (Fig 2A). In contrast, 32% to 41% of the ^{125}I–antithrombin III passed through the affinity column, and 50% to 56% of the load was recovered during NaCl gradient elution as a double peak (Fig 2B). Of the ^{3}H–antithrombin III loaded, approximately 70% passed through the column, and only 20% to 23% was bound by and recovered from Sepharose-heparin. This fraction eluted as a sharp peak (Fig 2C), but considerably sooner than unlabeled antithrombin III.

Sephadex G-200 gel filtration did not reveal any significant differences between either labeled or unlabeled antithrombin III preparations (data not shown). Furthermore, neither iodination nor reductive methylation caused a decrease in the antithrombin activity of the protein. Indeed, reductive methylation slightly increased the antithrombin activity by approximately 4% to 16%.

Binding of antithrombin III to the aorta wall. Samples of ^{3}H- or ^{125}I-labeled antithrombin III were taken up rapidly by the endothelium at 37 °C; binding of either labeled antithrombin III reached a plateau within two minutes (Fig 3A and B). In contrast, uptake by the subendothelium (intima-media layer) of normal aorta segments was almost linear for at least 20 minutes. Compared to uptake experiments conducted using 4% RSA-MEM, uptake of ^{125}I–antithrombin III by the intima-medial layer in the presence of 5 mmol/L Na citrate was decreased by 20% to 35%. In contrast, endothelium uptake was not influenced by Na citrate.

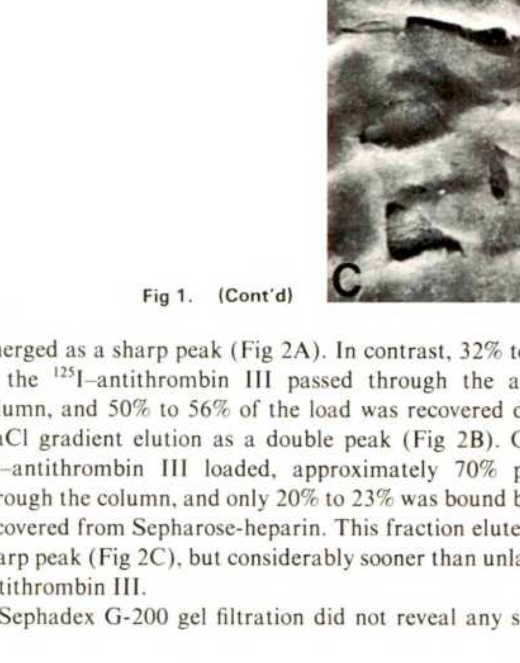

Fig 2. Chromatography of purified rabbit antithrombin III before and after radiolabeling on Sepharose-heparin (15 cm × 1.5 cm). (A) unlabeled, purified antithrombin III (approximately 3.5 mg), ●—● (B) ^{125}I–antithrombin III added to 100 mL of dialyzed supernatant from 50% saturated $(NH_4)_2SO_4$-treated rabbit plasma; absorbance (280 nm), □– – – –□; ^{125}I, ●—●. (C) ^{3}H–antithrombin III added to 100 mL of dialyzed supernatant from 50% $(NH_4)_2SO_4$-treated rabbit plasma; absorbance (280 nm), □– – – –□; ^{3}H, ●—●. After washing each column with 200 mL of the equilibration buffer (0.01 mol/L Na phosphate/0.15 mol/L NaCl, pH 7.3), the gradient (denoted by the arrow) was commenced: mixing chamber, 200 mL of equilibration buffer; limit solvent, 0.01 mol/L Na phosphate containing 1.5 mol/L NaCl). The fraction volume was 5 mL (ncpm, net count per minute).

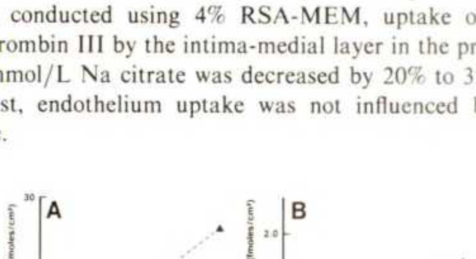

Fig 3. Uptake of radiolabeled antithrombin III by the luminal surface of normal aorta segments. (A) Segments were immersed in 1 mL 4% RSA-MEM containing ^{3}H–antithrombin III (42 pmol) for the times stated. After rinsing (one minute, 4 mL MEM), the vessel layers were stripped and counted. (B) Segments were placed in 1 mL 4% RSA-MEM containing ^{125}I–antithrombin III (1.3 pmol) and then treated as in (A). Antithrombin III was associated with both the endothelium (○—○) and subendothelium (intima-media) (▲—▲).

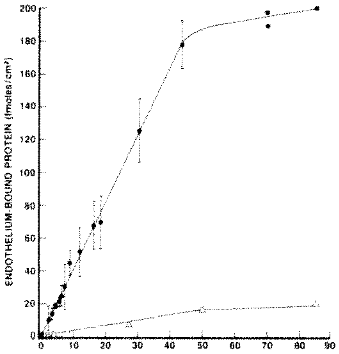

Fig 4. A comparison of binding of antithrombin III with binding of thrombin by the normal aorta endothelium. Aorta segments were placed for ten minutes in 1 mL 4% RSA (or BSA)-MEM containing up to 90 pmol of either thrombin (traced with ^{125}I-thrombin, ●—●) or antithrombin III (traced with either ^{125}I-antithrombin III, ○—○; or with ^{3}H-antithrombin III, △—△). After rinsing in MEM (one minute), the endothelial layer was stripped from each segment and counted. Thrombin results are given as the mean (± SD) where n = 6 to 11 aorta segments; each antithrombin III result is the average of two values.

Uptake of ^{125}I-antithrombin III (at a concentration of 1.3 to 3.2 pmol/mL) by the exposed intima-medial layer of deendothelialized (ballooned) segments during ten minutes (approximately 5.5 × 10^8 molecules/cm^2/min) was almost four times faster than that of ^{125}I-antithrombin III by the subendothelial layer of normal vessel segments (approximately 1.5 × 10^8 molecules/cm^2/min).

Comparison of uptake of antithrombin III with that of thrombin by the aorta endothelium. Each protein, in concentrations ranging from 1 to 60 pmol/mL, was incubated with intact aorta segments for ten minutes at 37 °C (Fig 4). These experimental conditions were assumed to allow equilibrium to be established between the endothelium-bound and free protein. Over the range of 10 to 60 pmol/mL, the uptake of thrombin by the endothelium was at least tenfold greater than the uptake of antithrombin III. The uptake of ^{3}H-antithrombin III did not appear to differ from that of ^{125}I-antithrombin III.

Effect of glycosaminoglycan-specific enzymes and heparin on the ability of the aorta wall to bind antithrombin III. Prior exposure of intact aorta segments to chondroitinase AC or chondroitinase ABC did not influence the uptake of ^{125}I-antithrombim III by either the endothelium or the underlying intima-media (Table 1). Heparitinase pretreatment did not affect uptake by the endothelium but did decrease uptake by the intima-media. Similar to the result with ^{125}I-antithrombin III, uptake of ^{3}H-antithrombin III was not significantly influenced by prior exposure of aorta segments to either chondroitinase AC or ABC. In comparison, the action of glycosaminoglycan-specific enzymes on the deendothelialized (ballooned) aorta segments caused a significant decrease in ^{125}I-antithrombin III uptake by the intima-medial layer. Indeed, heparitinase pretreatment reduced ^{125}I-antithrombin III to only approximately 20% of that of the control values.

Pretreatment of the normal vessel wall with heparin (10 U/mL) did not alter ^{125}I-antithrombin III uptake by either the endothelium or subendothelium. Furthermore, the quantity of endothelium-bound ^{125}I-antithrombin III displaced by either unlabeled antithrombin III, heparin, or thrombin was not significantly different from that displaced by 4% RSA-MEM (Table 2). However, ^{125}I-antithrombin III associated with the subendothelium of the normal vessel was actively displaced by either thrombin or heparin, and from the intimal surface of the ballooned vessel, by thrombin, heparin, or unlabeled antithrombin III.

Interaction between antithrombin III and thrombin at the normal vessel wall. In these experiments, either protein was labeled with ^{125}I radioactivity. When bound to the endothelium, ^{125}I-thrombin was rapidly displaced by placing the aorta segment in either purified antithrombin III or in defibrinogenated, recalcified rabbit plasma (Fig 5A): 50% of bound thrombin was displaced during five minutes (37 °C) by approximately 70 pmol of purified antithrombin III/mL of 4% RSA-MEM or by the equivalent of 35 pmol/mL of antithrombin III in recalcified plasma. The released radioactive products, when chromatographed on Sephadex G-200, gave similar elution profiles (Fig 5B and C). The products were of substantially greater molecular weight than either

Table 1. Effect of Pretreatment of Normal and Ballooned Aorta Segments With Various Agents on the Uptake of ^{125}I-Antithrombin III

Pretreatment		Percentage of ^{125}I-Antithrombin III Bound by		
		Normal Aorta		Ballooned
Reagent	Time (min)	Endothelium	Intima-Media	Intima-Media
4% BSA-MEM	5–10	100	100	100
Chondroitinase AC (5 IU/mL)	10	106	90	78
Chondroitinase ABC (5 IU/mL)	10	100	97	75
Heparitinase (5 IU/mL)	10	101	66	23
Heparin (10 U/mL)	5	114	86	—
Thrombin* (133 IU/mL)	10	101	73	—
Thrombin† (133 IU/mL)	10	142	109	—

Aorta segments were incubated for five or ten minutes in 1 mL of MEM containing BSA or RSA (4% wt/vol) and the reagent. The segments were rinsed (4 mL MEM, one minute) and then placed in ^{125}I-antithrombin III (usually 1.4 to 3.2 pmol, but see asterisks) in 1 mL of 4% BSA or RSA-MEM for ten minutes. The segments were rinsed (4 mL MEM, one minute), and then the layers of aorta wall, ie, the endothelium and the deendothelialized intima-media, were separated and the bound radioactivity measured. Control (ie, treatment with BSA or RSA-MEM alone) and test segments were always taken from the same aorta; mean results from test segments (n = 2 to 4) are compared to those from adjacent control segments and expressed as a percentage of bound radioactivity. For the two thrombin experiments, different antithrombin III concentrations were used: *1.4 pmol/mL, †0.14 pmol/mL.

Table 2. Displacement of ^{125}I–Antithrombin III Bound to the Normal and Ballooned Aorta Wall

Displacement		Percentage of ^{125}I–Antithrombin III Bound by		
		Normal Aorta		Ballooned Aorta
Reagent	Time (min)	Endothelium	Intima-Media	Intima-Media
None	—	100	100	100
MEM	5	64.1	61.2	—
4% RSA-MEM	2	55.5 ± 5.2	73.1 ± 15.4	—
4% RSA-MEM	5	45.9 ± 8.8	57.8 ± 9.4	83.2 ± 8.5
4% RSA-MEM	10	47.7 ± 9.4	57.5 ± 6.9	68.4 ± 7.7
Heparin (10 U/mL)	5	50.2 ± 6.2	37.1 ± 4.3	—
Heparin (10 U/mL)	10	—	—	28.3 ± 7.5
Antithrombin III (794 pmol/mL)	5	40.9 ± 7.8	55.2 ± 8.2	53.1 ± 7.0
Thrombin (67 IU/mL)	5	44.5	25.2	15.7 ± 5.9

Aorta segments were incubated in 1 mL 4% RSA-MEM containing ^{125}I–antithrombin III (1.6 to 3.2 pmol) for 10 minutes. After rinsing (4 mL MEM, one minute), the segments were exposed to various treatments to measure the displacement of bound ^{125}I–antithrombin III from the luminal surface of the vessel wall. As in Table 1, the endothelium and the deendothelialized intima-media were separated from the wall before counting; test and control (ie, no posttreatment) segments were taken from the same aorta, and results from test segments are expressed as a percentage (±SD, n = 4 to 8) of the bound radioactivity of the control results.

^{125}I–antithrombin III (Fig 5B) or ^{125}I-thrombin (Fig 5C) and, therefore, were presumed to contain thrombin–antithrombin III complexes.

DISCUSSION

Using an in vitro technique, we have shown that the capacity of the endothelium of the rabbit aorta for binding antithrombin III was considerably less than the capacity for binding either rabbit[13] or bovine thrombin at similar protein concentrations. Furthermore, unlike the endothelial binding sites for thrombin,[13,18] those for antithrombin III were not sensitive to glycosaminoglycan-degrading enzymes, and heparin pretreatment did not greatly influence the binding of ^{125}I–antithrombin III. Also, unfractionated ^{3}H–antithrombin III, which was shown to have a decreased affinity to Sepharose-heparin (Fig 2C) (due probably to *N*-methylation of the lysyl residues essential for heparin binding[1]), did not differ in its endothelium binding properties from that of unfractionated ^{125}I–antithrombin III (compare Figs 3A and 3B). Furthermore, uptake of unfractionated ^{125}I–antithrombin III by the aorta endothelium resembled that of the Sepharose-heparin–bound fraction of ^{125}I–antithrombin III.[18] Lastly, endothelium-bound antithrombin III, in contrast to endothelium-bound thrombin,[13] was not rapidly displaced by heparin. From these data we conclude that luminal binding sites for antithrombin III are relatively few in the normal aorta and that they are not of a proteoglycan nature. This conclusion is supported by the finding that pretreatment of the intact vessel segment with either purified antithrombin III or defibrinogenated plasma did not impede uptake of ^{125}I-thrombin or cause rapid formation of a ^{125}I-thrombin–antithrombin complex.[18] Also, our data compare well with those of Bauer et al[19] who compared the binding of ^{125}I–antithrombin III and ^{125}I-thrombin to cultured bovine endothelial cells.

In contrast to the endothelium, binding of ^{125}I–antithrombin III by the subendothelium of the intact aorta was rapid and nonsaturable over the time period of our experiments. The transendothelial uptake probably resulted from intercellular passage in a manner similar to that for ^{125}I-thrombin.[22] Thus, removal of the endothelium, ie, balloon catheter treatment, allowed the rate of uptake of ^{125}I–antithrombin III to increase substantially. Pretreatment of the deendothelialized (ballooned) surface with either chondroitinase AC, ABC, or, particularly, heparitinase decreased significantly the ^{125}I–antithrombin III uptake compared with the untreated, deendothelialized controls. Also, heparin actively displaced ^{125}I–antithrombin III from the deendothelialized vessel surface. We conclude that subendothelial glycosaminoglycans, particularly heparan sulphate, are responsible for much, if not all, of the ^{125}I-antithrombin binding in this region. This conclusion is supported by the observation that the Sepharose-heparin–bound fraction of ^{125}I–antithrombin III was bound three times more avidly by the deendothelialized (ballooned) intima-media than the unbound fraction (Hatton and Moar, unpublished results).

The significance of ^{125}I–antithrombin III uptake by the subendothelial layer of the intact aorta in vitro is not fully understood. Possibly, such an uptake indicates a compromised vessel surface that, lacking a normal, pressured blood flow, has relaxed, and the endothelial cells, instead of maintaining a flattened, plasma-tight surface, have become artificially parted from each other. The resulting intercellular gaps may encourage passage of certain proteins, particularly proteins with affinities to subendothelial constituents.

A portion (50% to 60%) of the antithrombin III bound to the endothelial surface of the vessel wall was readily displaced when incubated with 4% RSA-MEM (Table 2). Possibly, binding to the vessel wall may have resulted in endocytosis of the remainder of the antithrombin III. Neither heparin nor thrombin were able to dislodge more than 60% of the bound ^{125}I–antithrombin III (Table 2). The apparently poor reaction between endothelium-bound antithrombin III and thrombin may be explained by the following: (1) the quantity of displaceable antithrombin III bound to the endothelium is negligible; and (2) antithrombin III is bound at sites that are not of a glycosaminoglycan nature, and therefore, contact between bound antithrombin III and bound thrombin is unlikely. Thus, rather than antithrombin III binding to and becoming activated by pericellular proteo-

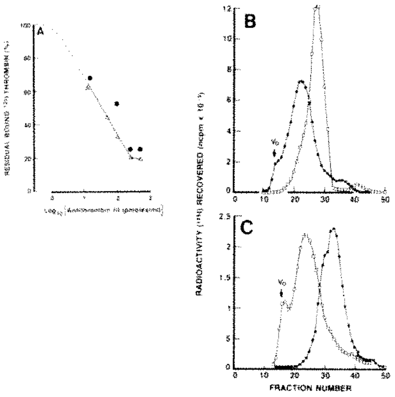

Fig 5. Displacement of ^{125}I-thrombin bound to the endothelium of intact aorta by either purified antithrombin III or defibrinogenated, recalcified rabbit plasma. (A) Aorta segments were exposed for ten minutes to ^{125}I-thrombin (1.7 to 5.3 pmol) in 1 mL 4% RSA-MEM; after washing in MEM (one minute), segments (containing 36.1 ± 21.2 femtomoles/cm^2 of endothelium) were placed for five minutes in either 1 mL 4% RSA-MEM containing antithrombin III, 1 mL of defibrinogenated plasma (diluted as necessary with MEM), or 1 mL of 4% RSA-MEM. The quantity of endothelium-bound ^{125}I-thrombin, after exposure to each antithrombin III concentration, is calculated as a percentage of the quantity of endothelium-bound ^{125}I-thrombin contained by adjacent aorta segments that had been exposed to 4% RSA-MEM only. Thus, ^{125}I-thrombin was displaced by purified antithrombin III (●) and by defibrinogenated plasma (Δ). The slope is extrapolated to the 100% value that represents the quantity of endothelium-bound ^{125}I-thrombin contained by control segments exposed to 4% RSA-MEM only. (B) Sephadex G-200 chromatography (58 cm × 1 cm) of the displaced products (●—●) obtained when aorta-bound, unlabeled thrombin (1-cm aorta segment exposed to 2,083 pmol of thrombin for ten minutes and then rinsed for one minute) was reacted with ^{125}I–antithrombin III (0.13 pmol) for five minutes. The radioactive products are compared with ^{125}I–antithrombin III (□—□) that was exposed to aorta that had been pretreated with 4% RSA-MEM only. (C) Sephadex G-200 (58 cm × 1 cm) elution profile of the soluble radioactive products (□—□) recovered from the interaction between aorta-bound ^{125}I-thrombin (1-cm aorta segment exposed to 0.2 μg of ^{125}I-thrombin for ten minutes and then rinsed for one minute) that had been exposed to Arvin-treated, recalcified rabbit plasma for two minutes. For comparison, ^{125}I-thrombin added to 150 IU of unlabeled purified thrombin as a carrier was chromatographed on a Sephadex G-200 column of similar dimensions (●—●).

glycan of the endothelium, a more likely anticoagulant property of the aorta endothelium stems from thrombin binding to proteoglycan and thus reacting with plasma antithrombin III.

Vessel wall–bound thrombin was rapidly displaced by antithrombin III or by defibrinogenated plasma (Fig 5A) to yield thrombin–antithrombin III complexes (Figs 5B and C).[16,18,22] The elution profile of the ^{125}I-thrombin–antithrombin III (Fig 5C) was similar to that of thrombin–^{125}I-antithrombin III (Fig 5B). Presumably, α-2-macroglobulin did not play a major part in thrombin inhibition by the defibrinogenated, recalcified plasma or a proportionately greater 19S peak would have been observed in Fig 5C as compared with 5B. However, we do not rule out the possibility of thrombin inhibition by other plasma protease inhibitors, eg, heparin cofactor II.[31]

Our observations on the relationship between antithrombin III and the endothelium contrast with other reports. Busch and Owen,[32] using both Langendorff heart preparations and cultured endothelial cells, concluded that the endothelium contained a surface cofactor, which was probably a proteoheparan sulphate, for binding antithrombin III. No direct measurement of antithrombin III binding was reported, and much of the evidence for a proteoglycan-containing binding site came from experiments using protamine and diisopropyl fluorophosphate (DFP)–thrombin as inhibitors of the heparin–antithrombin III and thrombin–antithrombin III reactions, respectively. However, these agents will also inhibit thrombin binding to the endothelium.[15,16,22] Marcum et al[4] have isolated a heparinlike anticoagulant from an endothelial cell fraction prepared from the miscrovasculature of calf retina. This glycosaminoglycan preparation was shown to bind to Sepharose–concanavalin A–immobilized antithrombin III and be displaced by 1 mol/L NaCl, but no mention was made whether thrombin would also bind to this fraction. The anticoagulant activity was destroyed by a purified heparinase preparation. More recently Marcum et al[3] demonstrated that perfusion of the microvasculature of the rat hind limb with heparinase destroyed the property that enhanced the rate of thrombin inactivation by antithrombin III. Perfusion of DFP-thrombin reduced the amount of the thrombin–antithrombin III complex formed by 30% to 40%, which confirmed earlier observations of Lollar and Owen.[15] These latter authors proposed that thrombin binding to the endothelium was a necessary step toward thrombin inactivation by antithrombin III.

The recent study by Stern et al[17] of bovine antithrombin III binding by bovine aorta segments would appear to contradict our observations on the rabbit aorta. However, the solvation technique used by Stern et al to quantitate the ^{125}I–antithrombin III bound to the endothelium differs significantly from the Hautchen technique[29] used in our study. In a preliminary investigation to compare these two procedures, we determined that the solvent (0.2 mol/L NaOH, 1% SDS, 0.01 mol/L EDTA) used by Stern et al dissociated 26% of the bound ^{125}I–antithrombin III from the endothelium and 54% from the underlying intima-media of normal rabbit aorta segments. We believe, therefore, that the relatively high values of ^{125}I–antithrombin III bound to the luminal surface that were reported by Stern et al are due to measurement of the ^{125}I–antithrombin III displaced from the endothelium and subendothelium, whereas with our procedure, endothelium-bound antithrombin III is sharply separated from that of the subendothelial compartment (Fig 1).

Possibly the endothelial cells of the microcirculation possess pericellular proteoglycans that act as binding sites for attracting antithrombin III and thrombin to catalyze thrombin inactivation. In contrast, the endothelial cells of the aorta attract only thrombin through proteoglycan-containing binding sites, and inactivation of endothelium-bound thrombin is performed by plasma antithrombin III. These two cellular mechanisms may have evolved to reflect different blood flow conditions. The microvasculature is comprised of 95% of the total vascular surface area but contains only 5% of the entire blood volume, which moves comparatively slowly (0.07 cm/s).

The arterial system contains <1% of the vascular surface area and transports approximately 20% of the blood at 40 to 50 cm/s.[33]

Finally, the presence of a significant pool of antithrombin III at the vessel wall is also supported by catabolic[34] and histologic studies,[35] although the histologic investigation of human blood vessels by Becker and Harpel[36] did not detect antithrombin III on the endothelial cell lining. However, if such a pool does exist, contained by pericellular heparinlike proteoglycan of the endothelium, then infusion of heparin would presumably displace this bound antithrombin III into the lumen, and a rapid rise in circulating anthithrombin III should be observed. To our knowledge, a heparin-stimulated increase in antithrombin III level has not been recorded; indeed, intravenous heparin therapy generally causes a decrease in antithrombin III levels in patients,[37-39] resulting probably from increased protein turnover.[40]

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Ionic Strength Dependence of the Polymer Solubilities of Deoxyhemoglobin S + C and S + A Mixtures

By Robert M. Bookchin and Tania Balazs

Factors contributing to the clinical differences between sickle cell–hemoglobin C disease (SC) and the benign sickle cell trait (AS) include the higher proportion of hemoglobin (Hb) S and the higher cell Hb concentrations in SC compared with AS red cells. Reports differ, however, about whether Hb C copolymerizes more than Hb A with Hb S when measured by minimum gelling concentrations (MGCs) and polymer solubilities of the deoxy-Hb mixtures. We now show that the MGCs and solubilities of equimolar mixtures of Hb S + Hb C vary much more with the ionic strength (μ) of the solution than those of Hb S + Hb A mixtures. At $\mu \leq 0.20$, but not at $\mu > 0.25$, Hb S + Hb C solubilities were significantly lower than those of Hb S + Hb A. These differences, which may reflect a greater effect of the β^6Lys$^+$ in Hb C at lower μ, can account for the reported discrepancies. The solubility differences were similar in the presence or absence of asymmetric hybrids, and since the intratetramerically cross-linked hybrids $\alpha_2\beta^s\beta^A$ and $\alpha_2\beta^s\beta^c$ had similar solubilities, they did not indicate the usual mechanism, involving greater incorporation of $\alpha_2\beta^s\beta^c$ into the polymers. The small solubility differences between the two Hb mixtures at physiologic (red cell) concentrations of Hb and 2,3-diphosphoglycerate probably play a minor role in the clinical differences between SC and AS states.

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SICKLE CELL–hemoglobin C disease (SC) is characterized by an overall clinical severity, in terms of the anemia and vasoocclusive episodes, that is intermediate between the severe homozygous sickle cell anemia (SS) and the essentially benign sickle cell trait (AS). Although earlier measurements of the minimum gelling concentrations (MGC) of hemoglobin (Hb) S-C mixtures[1-3] suggested that the major cause of the increased severity of SC disease, compared with AS, was a greater tendency of Hb C to copolymerize with Hb S, recent reports[4,5] have pointed out two other important factors contributing to SC severity—a larger proportion of Hb S in SC than in AS red cells and an increased intracellular Hb concentration in the SC cells. In one of these studies,[5] detailed measurements of polymer solubilities and the kinetics of polymerization showed no significant differences between comparable mixtures of Hb S + C and Hb S + A. No explanation was provided, however, for the apparent discrepancy between these results and the earlier MGC studies. A review of these different reports revealed that the solubility or MGC measurements were made in media whose ionic strength and composition varied considerably from one study to another. Since Hb C differs from Hb A by the substitution of the charged amino acid residue Lys$^+$ for the normal β6Glu$^-$, it seemed likely that any differences in the behavior of Hb C from Hb A would be dependent on ionic strength, ie, they would be minimized in relatively high ionic strength media that would shield the β6Lys$^+$ charge of Hb C. We therefore undertook a study of the ionic strength dependence of the MGCs and polymer solubilities of Hb S + A and Hb S + C mixtures.

Since the aforementioned studies also differed in the starting total concentration of Hb, which might itself affect the ionic strength of the mixtures, we also tested the effects on solubility of high initial total Hb concentration, as well as the effects of physiologic (red cell) levels of 2,3-diphosphoglycerate (2,3-DPG).

As will be discussed further, the location of the substitution in Hb C, which is the same as that in Hb S (β6) predicts that the β6 lysine residue in $\alpha_2\beta^s\beta^c$ asymmetric hybrid tetramers should not be in an intermolecular contact area of the Hb S polymer.[6,7] We therefore examined the role of $\alpha_2\beta^s\beta^c$ hybrids in polymerization by comparing the polymer solubilities of the nondissociable, intramolecularly cross-linked hybrid tetramers[8] $\alpha_2\beta^s\beta^c$, $\alpha_2\beta^s\beta^A$, and $\alpha_2\beta^s_2$. In addition, we compared the solubilities of Hb S + C mixtures in which hybrid formation was either permitted or prevented by deoxygenation of the non-S Hb prior to mixing.

MATERIALS AND METHODS

Fresh heparinized blood was obtained, with informed consent, from donors established to be homozygous for Hb S (SS) or for Hb C (CC) by electrophoretic and family studies at our Heredity Clinic and from normal (AA) donors. SS and CC donors were chosen in whom the proportion of Hb F in the red cell hemolysates, as measured by alkali denaturation,[9] was 2% or less. Hemolysates were prepared by the method of Drabkin,[10] and Hb concentrations were measured by the cyanmethemoglobin method.

Preparation of Hb solutions and mixtures. In some experiments Hb S was separated from the minor components Hb A_2 and Hb F by chromatography on columns of microgranular diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman), developed with 0.05 mol/L Tris-HCl, pH 8.1. Since the MGC and solubility results obtained using the purified Hb S did not differ significantly from those using unchromatographed, low-Hb F-hemolysates, subsequent experiments used the latter preparations. Since Hb A_2 cannot be readily separated from Hb C on a large scale[11] and attempts to isolate Hb C from AC or SC hemolysates would substantially enrich the proportion of Hb A_2 in the Hb C fraction with possible unintended effects on the solubilities,[12] CC hemolysates were used without further purification. Accordingly, for comparison no measures were taken to purify AA hemolysates. All hemolysates were dialyzed overnight at 4 °C against K-phosphate buffer (pH 7.35) between 0.025 and 0.15 mol/L. To maximize the accuracy of mixing equimolar quantities of Hb S + A or Hb S + C, in the majority of solubility experiments in which the Hbs were mixed in the oxygenated form, exact volumes of relatively dilute Hb solutions of measured concentration (about 10 g Hb/dL) were mixed prior to vacuum ultrafiltration and continued dialysis overnight against the appropriate buffers. The concentrated

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Hb mixtures were diluted with the same buffer to the desired starting total Hb concentrations. To avoid any possible effects of minor differences in Hb S hemolysates when comparing the MGCs and solubilities, each experiment compared Hb S + A and Hb S + C mixtures, each made in duplicate using the same Hb S solution.

Measurements of MGC and Polymer Solubilities. These procedures followed methods previously described.[8,13] Briefly, approximately 0.6 mL of Hb mixtures of measured starting total Hb concentrations were placed in 10-mL Ehrlenmeyer flasks and equilibrated for 30 to 40 minutes with water-equilibrated N_2 gas at 23 °C or in a warm room at 37 °C. The closed flask was briefly chilled on ice and, following anaerobic addition of a small volume of a deoxygenated solution of sodium dithionite in 0.001 mol/L NaOH to a final concentration of 1 mol/mol Hb tetramer, was rewarmed and the gas switched to dry N_2 to permit the Hb concentration to rise slowly by evaporation. The sample was chilled every few minutes to fully liquefy the thickening deoxyhemoglobin and to ensure complete mixing and rewarmed. Once a firm gel had formed, it was again chilled to liquefy it and, using a gas-tight syringe flushed with N_2 gas, transferred anaerobically to a cellulose nitrate tube under oil. A small aliquot was placed in a separate tube for measurements of Hb concentration (MGC). The centrifuge tubes were temperature equilibrated for 30 minutes and spun at 23 °C or 37 °C for 120 minutes at 122,000 g in a Beckman L3-50 centrifuge (Beckman Instruments, Inc, Fullerton, Calif) using an SW65K rotor to sediment the solid phase. The supernatants were fully decanted and aspirated into a separate tube and mixed completely before measuring the Hb concentration in triplicate, and the pH, using a glass microcapillary gun electrode (Radiometer, model E5021a Copenhagen), equilibrated at 23 °C or 37 °C. The ionic strengths of the phosphate buffer components were obtained using the nomogram of Boyd,[14] taking into account the final pH of the supernatants and, from the MGC values of total Hb concentration compared with the starting values, the effect of evaporation on the concentrations. The ionic strength of the sodium dithionite was calculated using the formula $\mu = \Sigma C_i Z_i^2/2$, where C_i is the molar concentration of each ionic component and Z_i its valency. To measure both the MGCs and solubilities on the same Hb solutions in these experiments for comparison with some of the earlier studies, the starting Hb concentrations were chosen to be about 1 g Hb/dL below the predicted MGCs; if gelation occurred either before dry N_2 gas was used or if the MGC exceeded the starting Hb concentration by more than 6%, the experiments were repeated starting with lower or higher total Hb concentrations, respectively.

In the experiments testing the effect on polymer solubility of initial Hb concentrations comparable to those in the red cell, it was not possible to measure MGCs, but otherwise the techniques just described were used. To test the effect of addition of 2,3-DPG, the Hb samples were dialyzed against 0.05 mol/L bis-Tris buffer, pH 7.35, containing 0.1 mol/L KCl (for controls without 2,3-DPG) or 0.08 mol/L KCl (for samples to which 2,3-DPG was added) so as to keep the final ionic strength similar. 2,3-DPG was added in solid form since the free acid (obtained by passing the pentacyclohexylammonium salt over Dowex 50 resin [Sigma Chemical Co, St Louis]) titrated to pH 7.0 with 0.1 N NaOH), thus giving a final concentration of 1 mol 2,3-DPG per mole Hb tetramer. An attempt was made to use a CO_2-bicarbonate buffer system instead of bis-Tris buffer, but in this system it was not possible to maintain equal final pH values in Hb S + C and Hb S + A solutions run simultaneously.

To prevent hybrid tetramer formation in Hb mixtures used to measure the MGC and polymer solubility, we followed a previously described[15] procedure based on the very small dissociation of deoxyhemoglobin tetramers into dimers. The non-S Hb (Hb A or Hb C) was deoxygenated in one flask, whereas the Hb S, which had been converted into the cyanmet-form to prevent gelation, was equilibrated with N_2 gas in another flask. An appropriate volume of the oxygen-free cyanmet-Hb S was then transferred anaerobically to the deoxygenated non-S Hb solution to achieve equimolar Hb quantities in the mixture, and after chilling and thorough mixing, the cyanmet-Hb was reduced to the deoxy-form by anaerobic addition of 1 mol sodium dithionite per mole cyanmetheme plus 1 mol dithionite per mole Hb tetramer. Parallel experiments were always done in which the Hb S and non-S Hb were mixed in the oxy-forms and the same additional amount of dithionite was added. The MGCs and polymer solubilities were then determined as previously noted. For these experiments, all Hb components were dialyzed and concentrated separately against 0.05 mol/L K-phosphate buffer, pH 7.35.

Preparation of cross-linked asymmetric hybrids. These preparations followed the methods of Macleod and Hill,[15] as described previously.[8,16] Equimolar mixtures of Hb S with either Hb C or Hb A in the CO-form were each reacted with the bifunctional cross-linking reagent difluoroditrophenylsulfone (FNPS), and the cross-linked tetramers were separated from unreacted Hb by chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) in 1 mol/L $MgCl_2$. Two of the three major components separated on preparative isoelectric focusing beds, representing cross-linked $\alpha_2\beta_2^s$ and either $\alpha_2\beta^A\beta^s$ or $\alpha_2\beta^s\beta^c$, primarily cross-linked between the NH_2 termini of the α chains,[15] were isolated, and stored in the CO-form as frozen beads in liquid N_2 until sufficient material was accumulated for solubility experiments. Then each was converted to the cyanmet-form to permit rapid conversion to the deoxy-form with dithionite, dialyzed, and concentrated against 0.05 mol/L K-phosphate buffer. The MGC and solubilities were measured as previously described except that the amount of dithionite added was 4.25 mole per mole Hb tetramer to provide an excess over the amount needed for reduction of the cyanmet-Hb.

RESULTS

Effect of ionic strength variations on solubilities of deoxyhemoglobins S + A and S + C. The polymer solubilities of equimolar mixtures of the deoxyhemoglobins S + A and S + C at 37 °C and 23 °C are shown in Fig 1. Although the ionic strength dependence of the solubilities of both mixtures was slightly greater at 23 °C than at 37 °C, the overall changes were similar at both temperatures, with Hb S + C mixtures showing a much larger ionic strength dependence than the Hb S + A mixtures. Although there was little or no solubility difference between the two mixtures in the higher ionic strength range, $\mu = 0.25$ to 0.40 (which includes the condition in 0.15 mol/L K-phosphate buffer), the Hb S + C mixtures were considerably less soluble than Hb S + A mixtures at ionic strengths below 0.20. Thus, in the ionic strength range 0.12 to 0.14 at 37 °C, the solubility of Hb S + A (26.8 g Hb/dL) was about 9% greater than that of Hb S + C (24.6 g Hb/dL).

In each of the experiments shown in Fig 1, MGC values were obtained following gelation of the Hb mixture prior to centrifugation. The Hb concentrations representing the MGCs (not shown) were consistently between 5% and 10% higher than the corresponding solubilities, as measured by the supernatant Hb concentrations after centrifugation. The patterns of change with ionic strength for Hb S + A and Hb S + C mixtures at both 25 °C and 37 °C were thus the same when comparing the MGCs as they were with solubilities.

Effects of high-Hb concentrations and 2,3-DPG on solubilities. Because of the possibility that Hb itself could contribute significantly to the total ionic strength inside red

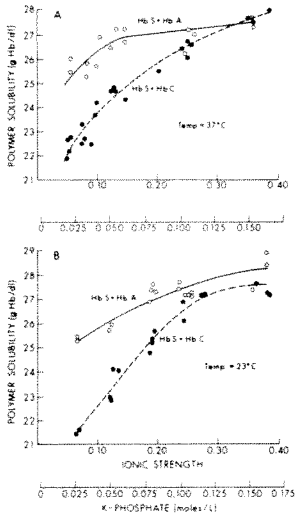

Fig 1. Effect of ionic strength variation on the polymer solubility of equimolar mixtures of Hb S + Hb A and of Hb S + Hb C. Each point represents the average of duplicate measurements using the same Hb mixtures, which differed from each other by 0.5 g Hb/dL or less. The final pH of the supernatant Hb solutions after centrifugation was 7.0 to 7.1. The ionic strengths of the solutions were calculated as described in Materials and Methods, taking into account the effects of evaporation on the final phosphate concentrations and the contribution of the sodium dithionite added. (A) 37 °C. (B) 23 °C.

cells (H.F. Bunn, personal communication), additional solubility measurements were made comparing deoxyhemoglobins S + A and S + C with initial total Hb concentrations comparable to those in the red cell, 34 g Hb/dL. In addition, the possible effects of the allosteric ligand 2,3-DPG were tested. The results (Table 1) indicate that the solubility differences between Hb S + A and Hb S + C are considerably smaller at these higher starting Hb concentrations than at initial Hb concentrations close to the MGC values. Solubilities in bis-Tris–KCl were similar to those in 0.05 mol/L phosphate buffer, but upon addition of 2,3-DPG, the differences between Hb S + A and Hb S + C were further reduced, although still statistically significant.

Table 1. Polymer Solubility of Hb S + A and Hb S + C Mixtures at Hb Concentrations Found in Red Cells

Buffer Condition	Solubility* (g Hb/dL) S + A	Solubility* (g Hb/dL) S + C	Mean Difference and Significance†
0.05 mol/L K-phosphate	32.83 ± 0.35	31.05 ± 0.36	1.78 ± 0.05 $P < .001$
0.05 mol/L bis-Tris 0.1 mol/L KCl	32.60 ± 0.16	31.17 ± 0.17	1.43 ± 0.06 $P < .001$
0.05 mol/L bis-Tris 0.08 mol/L KCl 5.0 mmol/L 2,3-DPG	32.87 ± 0.09	31.80 ± 0.14	1.07 ± 0.29 $P < .05$

*Each solubility value represents the mean ± SD of three or four paired samples in which Hb A or Hb C was mixed with the same solution of Hb S.

†Paired *t* statistic.

Starting total Hb concentrations were 34.0 g Hb/dL. All mixtures received, in addition, 1 mol sodium dithionite per mole Hb tetramer after equilibration with N_2 gas.

Table 2. Effect of Asymmetric Hybrid Tetramers on the MGC and Polymer Solubility of Hb Mixtures

Hb Components (Equimolar)	Hybrids Present MGC*	Hybrids Present Solubility*	Hybrids Absent MGC*	Hybrids Absent Solubility*
Hb S + Hb A	29.8	27.4	30.3	28.1
Hb S + Hb C	26.2	24.8	26.6	25.0

*g Hb/dL.

Each value represents the mean of a duplicate determination; duplicates differed by less than 0.5 g Hb/dL. The same solutions of Hb S, Hb C, and Hb A were used for these experiments.

Hemoglobins were dialyzed against 0.05 mol/L K-phosphate and mixed in the oxy-form for hybrid formation or deoxy- + cyanmet-forms to prevent hybrids prior to the addition of sodium dithionite, as noted in Materials and Methods.

Measurements were made at 23 °C. Final pHs of supernatants were 7.0 to 7.1.

Effects of asymmetric hybrid tetramers. To test the possible role of asymmetric hybrid tetramers (such as $\alpha_2\beta^s\beta^c$) on the solubility differences between Hb S + C and Hb S + A mixtures, we compared their MGCs and solubilities under conditions in which hybrid formation was permitted, by mixing the oxyhemoglobins prior to deoxygenation, or was prevented by maintaining the non-S Hb in the deoxy-state during mixing. The results, in Table 2, show that the solubility of Hb S + C, like that of Hb S + A, as reported previously,[16,17] showed no significant difference in the presence or absence of hybrids and that the lower solubility of the Hb S + C mixture did not depend on hybrid tetramer formation.

In addition, the solubilities of the hybrid tetramers $\alpha_2\beta^s\beta^c$ and $\alpha_2\beta^s\beta^A$ were compared directly, using the purified components cross-linked intratetramerically between their α chains to prevent dissociation. As shown in Table 3, under conditions in which the solubilities of Hb S + C mixtures were considerably lower than Hb S + A mixtures, the cross-linked hybrids $\alpha_2\beta^s\beta^c$ and $\alpha_2\beta^s\beta^A$ showed no significant differences in MGC or solubility.

DISCUSSION

The main findings of the present study are first, the observed larger ionic strength dependence of the solubility of

Table 3. MGC and Polymer Solubility of Intratetramerically Cross-linked (CL) Hemoglobins

Hemoglobin	MGC*	Solubility*
Native Hb S ($\alpha_2\beta_2^s$)†	22.2	—
CL-Hb S	26.6	25.4
CL-Hb SA ($\alpha_2\beta^s\beta^A$)	30.8	28.7
CL-Hb SC ($\alpha_2\beta^s\beta^C$)	31.6	30.2

*g Hb/dL.

†Value taken from ref 8 in which the same methods were used and shown for comparison.

Each value represents the mean of a duplicate determination; duplicates differed by less than 0.5 g Hb/dL. Measurements were made at 23 °C, and final pHs of supernatants were 7.0 to 7.1. See Materials and Methods for details.

deoxy-Hb S + C mixtures, compared with Hb S + A mixtures, explains the discrepancies in various reports about the behavior of the two mixtures. Secondly, under conditions mimicking the ionic strength and Hb concentrations within the red cell, the solubility of equimolar Hb S + C mixtures is only slightly lower than that of comparable mixtures of Hb S + A, substantiating the major conclusions of Bunn et al[5] that solubility differences between the two mixtures play no (or only a small) role in the clinical differences between AS and SC states. Thirdly, the solubility behavior of the asymmetric hybrids $\alpha_2\beta^s\beta^A$ and $\alpha_2\beta^s\beta^c$ are the same and are not the basis for the lower solubility of the Hb S + C mixtures at ionic strengths below 0.20.

Under conditions in which the gelation properties of deoxy-Hb S were commonly measured in phosphate buffers we find that both the solubilities and MGC values of equimolar mixtures of Hb S + C vary much more with the ionic strength of the media than do mixtures of Hb S + A. At ionic strengths below 0.20, the differences in MGC and solubility between the two Hb mixtures was fairly large, both at room temperature (23 °C) and at 37 °C, whereas the solubility differences disappeared at ionic strengths between 0.25 and 0.40. These findings can account for the discrepancies between earlier reports that the MGCs of Hb S + C mixtures were much lower than those of Hb S + A mixtures[1,2,3] and the recent report showing no differences.[5] Singer and Singer[1] made their measurements of MGC on undialyzed hemolysates diluted with distilled water or with buffers (whose concentrations were not described) and deoxygenated them with CO_2 gas. It is likely that the final solutions had a relatively low ionic strength as well as a relatively low pH. These investigators found the MGC values for Hb S + A mixtures from 12% to 20% higher than those of comparable Hb S + A mixtures. Allison,[2] who tested mixtures of Hb S + A and Hb S + C dialyzed against phosphate buffers, pH 7.35, ionic strength, 0.15 (equivalent to about 0.065 mol/L K-phosphate), also found both the MGCs and the minimum deoxy-Hb concentrations needed for a significant viscosity increment higher for the Hb S + A than for the Hb S + C mixtures. The results of Charache and Conley[3] are not easily compared with others since they studied the native hemolysates in which the proportion of HbS was 48% to 57% of the total Hb in the SC hemolysates and 34% to 41% in the AS hemolysates. Nevertheless, the large differences in the MGC values, 50% higher for the AS hemolysates, do not seem attributable only to the higher proportion of HbS in the SC cells and may be related to the way the solutions were prepared: these investigators diluted the hemolysates with distilled water before making their measurements so that the ionic strengths were probably below the physiologic levels. Cheetham et al,[18] who made their solubility measurements on Hb solutions dialyzed against phosphate buffer, pH 7.0, $\mu = 0.1$, plus 0.2 mol/L NaCl, which together give an ionic strength of $\mu = 0.3$, found no differences in the solubilities of equimolar mixtures of Hb S + A and S + C. Finally, Bunn et al[5] dialyzed the Hb solutions against 0.15 mol/L phosphate for which, at a final pH of about 7.0 to 7.1, $\mu = 0.38$; their addition of 0.055 mol/L sodium dithionite would have raised the value of μ to about 0.54. A possible complicating factor in the experiments of Bunn et al was that all the Hb components used were purified by chromatography on DEAE-Sephadex, which should separate Hb A_2 from Hb A or Hb S, but not from Hb C. Under these conditions and depending on whether the Hb C was obtained from homozygotes (CC) or heterozygotes (SC, AC), the proportion of Hb A_2 in the Hb C samples might be substantially enriched. For example, in the Hb C obtained from an AC hemolysate in which the proportion of Hb (C + A_2) averages about 41%,[19] the percentage of Hb A_2 may thus be increased from the normal 2% to 3% to more than 6%. Since admixture of Hb A_2 inhibits the polymerization of Hb S,[12,18] the solubility of Hb S + C mixtures might be unintentionally raised in relation to the Hb S + A mixtures. It is not possible to predict the precise effect of such enrichment of Hb A_2 on the solubilities since the solubility behavior of mixtures of three Hb components has not been studied. If we take the effect of Hb A_2 addition to Hb S to be similar to that of Hb F and if heterozygote (AC) blood was used to purify Hb C, an enrichment to 6% Hb A_2 would give about 3% Hb A_2 in a final 1:1 Hb S + C mixture. Extrapolation of the data of Sunshine et al[20] indicates that 3% Hb F would result in an increase of about 4.8% in the solubility of the mixture.

The ionic strength range over which the present measurements were made were intended to span the physiologic levels of ionic strength within the red cells as well as the higher levels at which some solubility measurements were made. Without allowing for the contribution of Hb, the main red cell ions, Na^+, K^-, Cl^-, and HCO_3^-, result in an ionic strength of $\mu = 0.12$.[21,22] It is difficult to assess the contribution of organic phosphates such as 2,3-DPG and ATP since they are variably bound to Hb (especially deoxy-Hb), but the total ionic strength, without Hb, should not exceed 0.13 to 0.14. No specific value can readily be assigned to the ionic strength contribution of Hb. Our findings that the solubility differences between mixtures of deoxyhemoglobins S + A and S + C are smaller at starting total Hb concentrations of 34 g/dL than at 26 g/dL, however, are consistent with the possibility that the contribution of Hb to the total ionic strength in the red cell is significant. The mechanism for the additional effect of a 2,3-DPG addition in raising the Hb S + C solubility towards that of Hb S + A is unknown. Although 2,3-DPG alone appears to have no clear effect on the solubility of deoxy-Hb S,[23,24] it could have a specific effect on the solubility behavior of deoxy-Hb C, alone or in the mixture.

Apart from the smaller solubility differences between the S + A and S + C mixtures at higher initial total Hb concentrations (34 g/dL), we also observed a large increase in the observed solubilities of both mixtures under these conditions compared with starting Hb concentrations of 26 g/dL. At least part of this increase can be attributed to enrichment of the sol phase with larger amounts of nonpolymerizing species. Nevertheless, our solubilities for both mixtures are considerably higher than those of Bunn et al[5] whose starting total Hb concentrations were nearly as high as ours. The most obvious difference between their experimental conditions and those of the present experiments is the much higher ionic strength of their solutions ($\mu = 0.54$ v 0.12

to 0.14 in the experiments of Table 1). It is conceivable that with increasing ionic strength beyond 0.4, the limit in our studies, the solubilities of these Hb mixtures fall. Another possible source of difference might be the solution pH: this was 7.0 to 7.1 in the present experiments, but in those of Bunn et al, the addition of 50 mmol/L dithionite would be expected to lower the pH further. At 38 °C, the decrease in deoxy-Hb S solubility with decreasing pH was reported to be quite steep.[25] It was beyond the scope of the present studies, however, to test all these possibilities or to try to reproduce the exact experimental conditions of various investigators.

Previous studies of the interactions of Hb S with other Hb types or mutants indicated that facilitation or inhibition of gelation, in relation to Hb S + A mixtures, was mediated by the formation of asymmetric hybrid tetramers of the type $\alpha_2\beta^s\beta^x$ (or $\alpha_2\beta^s\gamma$, $\alpha_2\beta^s\delta$).[16,26] One or more amino acid residues in the trans–β chain (not containing the β6Val contact site) could then be implicated as a probable intermolecular contact in the deoxy-Hb S polymer. The lower solubility of deoxy-Hb S + C mixtures (compared with deoxy-Hb S + A) appeared paradoxical in this context since considerable evidence both from gelation studies with Hb mixtures[6] and from studies of the deoxy-Hb S crystal double strand[7] seemed to exclude involvement of the NH_2 terminal region of the trans–β chains in polymer contacts. The present results, showing no differences in the solubility of deoxy-Hb S + C mixtures with and without hybrid formation and essentially the same solubilities of the cross-linked hybrids $\alpha_2\beta^s\beta^c$ and $\alpha_2\beta^s\beta^A$, suggest that the lower solubility of the Hb S + C mixtures occurs by a mechanism independent of asymmetric hybrid formation and therefore different from the mechanism underlying the observed behavior of Hb S with several other mutants.[16,26] It was observed earlier in this laboratory[27] that the MGC of both Hb S and Hb A + S mixtures varied with the ionic strength of the medium and was lowest at very low ionic strength. The MGC of deoxy-Hb S varied similarly with ionic strength (not with molarity) in potassium phosphate and in NaCl or KCl. These findings were interpreted to suggest that electrostatic bonds are involved in deoxyhemoglobin S polymerization as well as the hydrophobic interactions suggested by the negative temperature coefficient of gelation. Our findings of a large ionic strength dependence of the solubility of deoxy-Hb S + C mixtures suggest that the difference from Hb S + A mixtures somehow depend on the charge of the substituted residue in Hb C, β6Lys$^+$.

If asymmetric hybrid tetramer formation is not involved in the solubility-lowering effect of Hb C in deoxyhemoglobin S + C mixtures, the question remains as to what is the mechanism of the different behavior of Hb C and Hb A. One possibility is that deoxy-Hb C itself may crystallize to some extent under the conditions of these experiments. Such a possibility was considered by Bunn et al[5] as an explanation for their findings that in Hb S + C mixtures containing more than 60% Hb C the solubilities were slightly lower than those expected from their copolymerization model. The solubilities of deoxy- and CO-Hb C and Hb A have been compared, however, only at ionic strengths of 0.6 or higher[28]; at $\mu = 0.6$ (in phosphate buffer, pH 7.2), deoxy-Hb C was considerably less soluble than the CO-forms. Thus, some deoxy-Hb C might crystallize, lowering the overall solubility of the deoxy-Hb S + C mixtures, but this possibility would require further measurements of the solubilities of Hb C and Hb A at lower ionic strengths.

Another possible mechanism of the lower solubility of deoxy-Hb S + C mixtures that does not involve asymmetric hybrids could be that some tetrameric Hb C might be incorporated into the Hb S polymers. Incorporation of non-S tetramers, both Hb A and Hb C, was suggested earlier by Allison[2] and Bertles et al,[29] the latter based on evidence of incorporation of both deoxyhemoglobins A and C into the solid phase of centrifuged gels of Hb S + A and Hb S + C mixtures. Although some more recent analyses have calculated that non-S Hb tetramers, unlike hybrid tetramers containing a β^s chain, are excluded from the polymer phase,[29,30] the opposite conclusion was reached by other investigators,[17,31] and more direct analyses of the polymer composition will be required to resolve this question.

Since the differences in solubility between deoxyhemoglobins S + C and S + A were quite small when measured at starting total Hb concentrations found in red cells and were reduced further upon addition of physiologic levels of 2,3-DPG, the present results appear to substantiate the major conclusion of Bunn et al[5] that the main causes of the increased sickling tendency and greater clinical severity of sickle cell–Hb C disease over sickle cell trait are the higher proportion of Hb S and higher intracellular Hb concentrations occurring in S + C red cells. The small solubility differences between Hb S + C and Hb S + A expected to occur with red cell Hb and 2,3-DPG concentrations must play a minor role in determining clinical severity.

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Separation of the Acetylcholinesterase-Deficient Red Cells in Paroxysmal Nocturnal Hemoglobinuria

By Feng-Lan Chow, Sharon E. Hall, Wendell F. Rosse, and Marilyn J. Telen

Blood of patients with paroxysmal nocturnal hemoglobinuria (PNH) most often contains two or more populations of erythrocytes—one population with normal sensitivity to lysis by complement (PNH I cells) and a second population of moderately abnormal cells (PNH II cells) or markedly abnormal cells (PNH III cells). PNH II and III cells exhibit moderately and markedly increased sensitivity to lysis by complement, respectively, as well as other membrane defects. We have devised a method for isolating pure, intact PNH II and III cells from mixed populations by use of monoclonal antibodies and cell affinity chromatography. Study of purified cell populations has led to the identification of a further subtype, PNH IIIb, of PNH erythrocytes. PNH IIIb erythrocytes are less sensitive to complement lysis than PNH IIIa cells but are lysed by fluid-phase activation of complement, unlike PNH II erythrocytes.
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PAROXYSMAL nocturnal hemoglobinuria (PNH) is characterized by red blood cells that are abnormally sensitive to the hemolytic action of complement.[1,2] This abnormality is usually present in some but not all red cells; thus, populations of red cells with different sensitivity to the hemolytic action of complement are present in the circulation. Three degrees of sensitivity to complement have been described[3,4]: some of the red cells may be normal (PNH I cells), some may be moderately (two- to fourfold) more sensitive than normal (PNH II cells), and some may be markedly (15- to 25-fold) more sensitive than normal (PNH III cells). These populations occur in various combinations and various proportions in different patients with PNH. It is this mixture of cells that has made it difficult to analyze the membrane defect leading to increased lysis by complement.

In 1960, Auditore et al found that the activity of the enzyme acetylcholinesterase was variably deficient on the red blood cells of patients with PNH.[5] Kunstling and Rosse demonstrated that it was the cells that were abnormally sensitive to the lytic action of complement that were deficient; the normal PNH I cells appeared to have normal amounts of the enzyme.[6] Recently, Chow et al demonstrated by the use of monoclonal antibodies to the enzyme that the protein immunologically characteristic of the enzyme was lacking in these cells.[7] In the present paper, we demonstrate how these facts have been used to devise a technique to separate the complement-sensitive from the complement-insensitive red cells in the blood of patients with PNH. We have used this technique to define a fourth variant population in PNH red blood cells, a subpopulation of PNH III cells that are slightly less sensitive to complement lysis than the majority of PNH III cells.

MATERIALS AND METHODS

Red blood cells. Blood from normal donors or patients with PNH was stored under sterile conditions in Alsever's solution.[8] At the time of use, the erythrocytes were washed in phosphate-buffered saline (PBS), pH 7.4, and adjusted to a concentration of 2×10^8/mL.

Antibodies. Monoclonal antibodies (AE-1, AE-2, AE-3, and AE-4) made by mouse hybridomas against four different epitopes of the acetylcholinesterase molecule were the kind gift of Dr D. Fambrough[9] in the form of purified IgG. In addition, AE-1 and AE-2 hybridoma cells were obtained from American Type Culture Collection and grown as ascites tumors in BALB/c mice. P3 (P3x63/Ag8), the immunoglobulin product of a murine myeloma cell line, was also grown as murine ascites tumors and used as an isotype specific (IgG1) control for the reactions of the AE monoclonal antibodies.

Antibody to mouse IgG made in rabbits was obtained as affinity-purified IgG from Cappel Laboratories, Malvern, Pa. This was shown to react with monoclonal antibody attached to red blood cells by bindng of radiolabeled staphylococcal protein A (SPA).

Complement lysis sensitivity tests. The sensitivity of human red cells to the lytic action of complement was determined by a modification of the complement lysis sensitivity (CLS) test of Rosse and Dacie.[3] The total reaction volume was reduced to 375 μL, but the proportions and materials remained the same. The value used to compare sensitivity of different cell populations to lysis by complement is the CLS H_{50}, defined as the reciprocal of the dilution of serum used (270 in these studies) divided by the milliliter equivalents of serum required to obtain lysis of 50% of the cells.

Binding of monoclonal antibodies to normal red cells. To determine which monoclonal antibodies best bound to the acetylcholinesterase on normal cells, equal volumes (100 μL) of a 5% suspension of washed normal red blood cells and a saturating dilution of monoclonal antibody (or a comparable dilution of control ascitic protein [P3]) were incubated at room temperature for 30 minutes. The cells were then washed twice, brought to a volume of 100 μL, and incubated with an equal volume of rabbit antimouse IgG (2 mg/mL diluted 1:50) at room temperature for 30 minutes. After washing, the cells were again suspended in 100 μL and incubated with 100 μL of ^{125}I-labeled SPA (8×10^4 cpm). Triplicate aliquots of 50 μL each were layered on a mixture of phthalate esters (1.5 parts *N*-butyl phthalate, Fisher Scientific Co, Pittsburgh, Pa to one part bis-2-ethylhexyl phthalate, Eastman Kodak Inc, Rochester, NY) in plastic microcentrifuge tubes and centrifuged in a Beckman microfuge (Beckman Instruments, Fullerton, Calif). The cells were removed by clamping and cutting the tube, and the radioactivity was counted in a well-type scintillation counter.

Separation of red blood cells on a column. Washed red cells (1×10^8/mL) were incubated with equal volumes of each monoclonal antibody (AE-1 and AE-2) at a dilution that saturated all

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sites for 30 minutes at room temperature on a rocker platform. They were washed by centrifugation, suspended to the original volume, and incubated with an equal volume of rabbit antimouse IgG (1:50) for 30 minutes at room temperature on a rocker. After two washes, they were pipetted onto the top of the separation column.

The separation column consisted of 10 mL of Sepharose 6MB to which SPA had been covalently attached (obtained from Pharmacia Fine Chemicals, Piscataway, NJ). The column was packed by gravity and washed thoroughly with PBS buffer containing 1% bovine serum albumin before each use.

After application of the cells, the flow rate was adjusted to about 0.2 mL/min; this flow rate was maintained until no further cells could be eluted from the column (usually about 50 minutes). The flow rate was then increased to 3 to 4 mL/min again until no further cells were eluted.

The column was regenerated by thorough (ten-bed volume equivalents) washing with 0.1 mol/L glycine-HCl buffer, pH 3, containing 0.1 mol/L NaCl. It was then thoroughly washed with PBS and stored at 4°C in 0.1 mol/L phosphate buffer with sodium azide (0.05% w/v) pH 6.0.

RESULTS

Binding studies. As shown in Table 1, the monoclonal antibodies AE-1, AE-2, and AE-3 all bound via rabbit antimouse IgG about the same amount of radiolabeled SPA to red cells; AE-4 bound less under similar conditions. Studies using AE-2 monoclonal antibody and radiolabeled $F(ab')_2$ antimouse IgG (Amersham Corp, Arlington Heights, Ill) showed that maximum binding of the radiolabeled anti-IgG was ten to 50 molecules per cell, indicating an extremely low density of antigen. No great increase in binding of radiolabeled SPA was found with combinations of monoclonal antibodies except in the case of the mixture of AE-1 and AE-2. For this reason, this combination was used for the chromatographic separation experiments.

Chromatography of normal red blood cells. When normal red blood cells sensitized with AE-1 and AE-2 and rabbit antimouse were chromatographed on the Sepharose 6MB-SPA column, no cells were eluted during the first (slow flow) phase; 80% ± 5% of the cells were eluted during the rapid flow phase. Cells not sensitized or sensitized with P3 and treated in the same way were eluted during the first (slow) phase (80% ± 10%). This indicated that red blood cells that had the acetylcholinesterase enzyme and that had been reacted with the antiacetylcholinesterase monoclonal antibodies were specifically retained on the column under these conditions.

Table 1. Binding of SPA to Normal Erythrocytes by Monoclonal Antibodies to Acetylcholinesterase and Rabbit Antimouse IgG

Antibody*	^{125}I-Labeled SPA (cpm Bound)
AE-1	5,491
AE-2	5,777
AE-3	5,115
AE-4	1,254
AE-1 + AE-2	7,319
AE-1 + AE-3	6,612
AE-1 + AE-4	6,077
AE-2 + AE-3	6,557
AE-2 + AE-4	6,037
AE-3 + AE-4	6,098
P3 control	389

*All antibodies used in saturating amounts, as determined by binding of radiolabeled antimouse Ig.

To test the ability of the column to separate antibody-coated cells from those not coated by antibody, type A red cells from a normal donor were coated with AE-1 plus AE-2 and rabbit antimouse IgG; type O cells from a normal donor were reacted with the control protein, P3. The cells were

Fig 1. **Separation of the populations of PNH cells by affinity chromatography. After incubation with monoclonal antibodies to acetylcholinesterase and rabbit antimouse antibody, PNH red cells were applied to a column containing Sepharose 6MB to which SPA was covalently attached. The PNH I cells bearing acetylcholinesterase were retarded (upper band), whereas those lacking the enzyme (PNH III cells) passed through the column (lower band).**

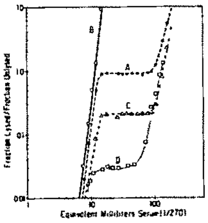

Fig 2. Analysis of the sensitivity to complement lysis of PNH I and PNH III cells separated by affinity chromatography. The analysis of the CLS of the unseparated cells is shown in curve A; 50% of the cells were PNH I, and 50% were PNH III. The analysis of the CLS of the cells eluted from the column by slow flow rate is shown in curve B; these cells were entirely PNH III cells since the CLS curve forms a straight line. Results of the CLS of the cells retained on the column in the first passage are shown in curve C; the proportion of PNH I cells was increased from 50% to 83.3%. When these cells were rechromatographed, the proportion of PNH I cells in the retained cell population increased to 97.1%, as shown by curve D.

mixed and chromatographed through the column. Two separate populations were evident during the slow elution phase, with only type O cells eluted during this phase as shown by lack of agglutination of the eluted cells with anti-A. The type A cells were eluted during the phase of rapid flow.

Chromatography of PNH red blood cells. When PNH red blood cells of the common phenotype (PNH I and PNH III cells) were treated in this way, there was a separation on the column into two distinct populations (Fig 1): one population eluted from the column during the first (slow) phase of the elution process, the other eluted during the rapid elution phase. When these separated cells were tested in the CLS test, those that were not adherent to the column were entirely PNH III cells (Fig 2, line B). Those that were adherent were a mixture of the two populations but were enriched for PNH I cells (Fig 2, curve C). If the cells retained on the column and eluted with rapid flow were again passed through the column, they were further enriched for PNH I cells (Fig 2, curve D), indicating that the contaminating PNH III cells did not remain on the column because of a specific reaction with the column.

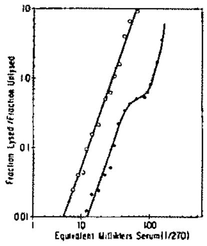

Fig 3. Analysis of the sensitivity to complement lysis of PNH I and PNH II cells separated by affinity chromatography. The analysis of CLS of the unseparated cells is shown in solid symbols; 66% of the cells were PNH 1, and 34% were PNH II. The analysis of CLS of the cells eluted from the column by slow flow rate is shown in open symbols; these cells were entirely PNH II cells. The CLS of the PNH II cells was not changed by passage through the column. Lysis of 50% of the purified PNH II cells occurred at approximately 29-mL equivalents of serum, whereas lysis of 50% of the PNH II cells within the unseparated cell suspension occurred at approximately 35-mL equivalents of serum.

Table 2. Complement Sensitivity of Affinity-Purified PNH III Red Blood Cells

Patient	Percentage of PNH III in Whole Blood	Analysis of Cells Lacking Acetylcholinesterase: PNH IIIa Percentage	PNH IIIa CLS H_{50} Units*	PNH IIIb Percentage	PNH IIIb CLS H_{50} Units*
V D.	73	65	36	35	19
J P	58	65	31	35	17
W.M	28	49	67	51	18
J.G.	44	69	38	31	16
O P.	42	100	27	—	—
E.D.	47	100	24	—	—
J.B.	37	100	37	—	—
E.G.	61	100	41	—	—
A.H.	15	100	41	—	—
M.F.	60	100	27	—	—
C.K.	>95	—	—	>95	16

*CLS H_{50} is quantitated by determining the reciprocal of the dilution of serum containing a normal amount of complement, 1 mL of which would lyse 50% of the sensitized cells of a population in a total volume of 7.5 mL. Normal CLS is 2 0 to 3 2 CLS H_{50} units

When cells containing PNH I and PNH II cells were treated as previously described and passed through the column, the PNH II cells were also eluted during the slow elution phase. The cells that were eluted during the slow elution phase were pure PNH II cells (Fig 3).

The use of the column to define a subpopulation of PNH III cells. The red cells of 11 patients who were defined as having PNH I and PNH III cells by conventional CLS testing were separated on the column by the aforementioned procedure. When the red cells lacking acetylcholinesterase were tested in the CLS test, six of 11 were found to be of a single population in that the analysis demonstrated a single straight line (as in curve B, Fig 2). However, in four of 11 patients, there appeared to be a second population of cells that were slightly less sensitive than the PNH III cells as previously defined (Table 2 and Fig 4). The cells of one patient appeared to consist entirely of these slightly less sensitive cells (Table 2). When the PNH III cells from these four patients were isolated by affinity chromatography and lysed by complement activated in the fluid phase by cobra venom factor, 100% of the PNH III cells from each patient were lysed. Thus, the slightly less complement-sensitive

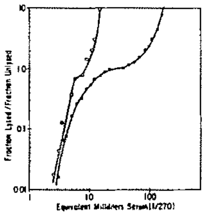

Fig 4. Analysis of the sensitivity to complement lysis of PNH I, PNH IIIa, and PNH IIIb cells separated by affinity chromatography. The analysis of CLS of the unseparated cells is shown in solid symbols; 50% of the cells were PNH I, and 50% were PNH IIIa + PNH IIIb. The analysis of CLS of the cells eluted from the column by slow flow rate is shown in open symbols; 43% of these cells were PNH IIIa cells, and 57% were PNH IIIb cells.

PNH III cells did not resemble PNH II cells in this respect.[10] We have named these intermediate cells PNH IIIb cells and the more complement-sensitive, common variant, present in ten of 11 examples, PNH IIIa cells.

DISCUSSION

One of the facts that has made it difficult to search for the nature of the membrane defect in PNH red cells that renders them susceptible to the lytic action of complement is the existence, in most patients, of a mixture of red cell populations. Thus, the results of biochemical analyses have depended upon the proportion of abnormal cells present, and it has been difficult to define which of the observations were pertinent to abnormal cells. In the past, this difficulty has been minimized by the use of cells of patients with large porportions of abnormal cells.[11-13] However, such patients are rare, and their rarity makes statistical analysis of findings derived from them difficult in that comparisons among patients cannot be readily made.

Previously, PNH I and PNH II cells have been separated from PNH III cells by differential lysis.[14] PNH III, but not PNH I or PNH II, red cells bind the membrane attack complex when it is initiated in fluid phase by cobra venom factor and are lysed by this process.[10] The PNH III cells in a mixture of cells are lysed by cobra venom factor and serum, and the surviving non–PNH III cells can thus be purified. However, the surviving population(s) (usually PNH I cells) is often the one that is not under investigation. Further, the membranes of the populations that are lysed by complement are contaminated by the presence of complement components; this may interfere with the analysis of their structure.

Suspensions of intact cells enriched by complement-sensitive cells can be obtained by differential centrifugation since the proportion of abnormal cells tends to be greater in the young and hence less dense cells.[3] This is because the proportion of abnormal (PNH III) cells that are delivered to the circulation is much greater than the proportion of these cells in the peripheral blood.[15] However, the enrichment for PNH III cells by centrifugation is always incomplete, and the abnormal cells are contaminated by the normal PNH I cells.

The technique described in this paper allows for the first time the separation of the abnormal cells in PNH from the normal PNH I cells by taking advantage of the fact that these abnormal cells lack the protein for the enzyme acetylcholinesterase.[7] Since the antibodies that are used in these studies do not react with the abnormal cells, there is little or no contamination of the membrane with immunoproteins and no disruption of membrane structure. The cells are isolated whole, and the characteristics of lysis in response to complement activation can be analyzed. Further, the presence of the monoclonal antibodies to acetylcholinesterase and antimouse IgG has no effect on the sensitivity of the PNH I and normal red blood cells to complement lysis. This is probably because the number of copies of the antigen is so small compared to the number of copies of the antigen against which the anti-I antibody reacts in this test (probably greater than 600,000 per cell).[16] It is also the low density of acetylcholinesterase molecules[7] that allows normal and PNH I cells to be eluted from the column by increased flow rate alone, without changes in pH or the addition of denaturing agents.

The advantages of such separations in the analysis of the abnormalities of the red cells in PNH are illustrated in the ability to define a new subpopulation of PNH III cells. These cells are so little different from the usual PNH III cells that they were not detected in the analysis of complement sensitivity of mixed populations of red cells. Only when the abnormal cells were separated did the difference become apparent. Nevertheless, the identification of these cells has clarified the interpretation of the CLS curves of some patients.

We have also used this technique to separate the abnormal cells for analysis of the presence of decay-accelerating factor (DAF), a membrane protein that normally controls the formation and activity of the C3 convertases of complement.[17] This protein is completely or partially lacking in the complement-sensitive red cells of PNH.[18,19] By the use of separated populations, we have been able to demonstrate the effects of the absence of DAF and of the repletion of DAF purified from normal cells.[20] The nature of the relationship between acetylcholinesterase deficiency and DAF deficiency is currently under investigation.

By the use of the techniques described herein, purified populations of PNH cells can be obtained from nearly all patients with PNH. This will markedly simplify the analysis of these cells for the nature of their membrane defect(s) and will allow more ready comparison of the cells of different patients.

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Analysis of Leukocyte Differentiation Antigens in Blood and Bone Marrow From Preleukemia (Refractory Anemia) Patients Using Monoclonal Antibodies

By Peter Hokland, Gitte Kerndrup, James D. Griffin, and Jørgen Ellegaard

Peripheral blood and bone marrow mononuclear cells from patients with refractory anemia (RA) or RA with sideroblasts (defined according to the revised French-American-British classification with less than 5% blast cells in the bone marrow) were analyzed using a panel of monoclonal antibodies directed against leukocyte antigens on B lymphocytes, T lymphocytes, monocytes, and myeloid cells. In the peripheral blood an increased proportion of T lymphocytes (and correspondingly a decreased proportion of B cells) could be demonstrated. However, when expressed in terms of absolute numbers, the T cell component was depressed because of severely decreased numbers of T4+ helper cells. In contrast, the absolute numbers of T8+ suppressor cells were either normal or increased in the majority of the patients. This resulted in markedly decreased ratios of T4+/T8+ cells, which were closely correlated to the number of transfusions given to the patients because of their refractory anemia. Finally, nearly all of the patients exhibited decreased numbers of cells reactive with the N901 natural killer (NK) antibody, thus explaining our earlier finding of decreased NK activity in these patients. In the bone marrow increased proportions of myeloid cells reactive with monoclonal antibodies present on immature myeloid cells (My7 and My9) were found, suggesting the presence of malignant clones. Indeed, when the numbers of My7+ cells and the morphologic evaluations of bone marrow smears at the time of diagnosis were compared to the progression of the disease, a group of patients with high numbers of My7+ cells and normal morphology could be identified that had a high probability of progression to refractory anemia with an excess of blasts or to overt acute myeloid leukemia. Thus, the use of antibodies defining leukocyte differentiation antigens might be of significant value in the diagnosis and prognostication of the myelodysplastic syndromes. These findings are discussed in relation to the pathogenesis of this potentially premalignant condition with special emphasis on possible defects in the immunologic defense mechanisms against early neoplasias.

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ACUTE MYELOID LEUKEMIA (AML) is in some patients preceeded by a myelodysplastic phase in which varying degrees of bone marrow dyshematopoiesis are dominant features. The term myelodysplastic syndrome includes five subgroups distinguished by their different percentages of blast cells in the bone marrow and peripheral blood according to the recent revision of the French-American-British classification of acute myeloid leukemias.[1] There, refractory anemia (RA) is defined as a condition with fewer than 5% myeloblasts in the bone marrow, and this strengthening of the diagnostic criteria has had the advantage of defining a more homogeneous group of patients in which potentially premalignant processes can be studied.

Even though much is known about the growth characteristics[2] and cytogenetics of bone marrow cells,[3] information about the immune system in these patients is still scarce. Since several parameters of the immune system have been envisaged to play a role in the defense against early neoplasias, this condition seems to be well suited for elucidating immunologic alterations in potentially premalignant conditions We have previously demonstrated that patients fulfilling the new and more strict criteria for preleukemia exhibit a markedly diminished natural killer (NK) cell activity in peripheral blood, probably because of a low number of circulating NK cells.[4] Here we describe the results from measurements of leukocyte differentiation antigens on blood and bone marrow mononuclear cell suspensions from such patients using a panel of monoclonal antibodies (MoAb) suited for evaluating both lymphocyte subsets as well as myeloid cells. Although these patients were found to be heterogeneous with respect to antigen expression, we were able to detect several abnormalities of potential interest in relation to the prognosis of this group of patients.

MATERIALS AND METHODS

Patients. Thirty-two patients, 17 females and 15 males, were found to fulfill the diagnostic criteria of RA discussed in detail elsewhere[5]; ten patients presented with ring sideroblasts (RA-S). Fifty-five percent had clonal karyotypic abnormalities when examined by chromosome-banding techniques.[6] It should be stressed that all had less than 5% blast cells in the bone marrow and none had myeloblasts in the peripheral blood at the time of diagnosis.

Preparations of mononuclear cell suspension from peripheral blood and bone marrow Freshly drawn, heparinized venous blood was layered onto Isopaque-Ficoll (Nygaard, Oslo), and red cells and mature myeloid cells were removed by density centrifugation.[7] The interface cells were washed twice in Hanks' buffered salt solution (HBSS) supplemented with 2.5% pooled human AB serum and adjusted to a concentration of 1 × 10^6/mL. Bone marrow was suspended in preservative-free heparin and diluted approximately 1:4 in HBSS After Isopaque-Ficoll centrifugation the cells were treated as previously described for peripheral blood cells.

Monoclonal antibodies. The MoAb used in this study are shown in Table 1 together with a summary of their reactivities and original references They were selected to give as complete a picture of the differentiation antigens on T cells, B cells, monocytes, and myeloid cells as possible. B1 (provided by Dr L.M. Nadler) is expressed on B cells and some malignant B cells including 50% of acute lymphoblastic leukemia and 40% of lymphoid blast crisis cells of chronic myeloid leukemia. For the definition of the T3 pan/T cell antigen, the UCHT-1 antibody produced by Dr P.C. Beverley was used. The T4 helper/inducer antigen and the T8 cytotoxic/suppressor antigen were defined by the B.66.6 and the B.99 1 antibodies produced by Dr

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Table 1. Monoclonal Antibodies Used in This Study

Antigen	MoAb	Reactivity in Normal Donors	Reference
T3	UCHT-1	mature T cells	8, 9
T4	B.66.6	helper/inducer T cells	9, 10
T8	B.99 1	suppressor/cytotoxic T cells	9, 10
B1	anti-B1	immature and mature B cells	11
HNK-1	N901	NK cells	12
My4	anti-My4	monocytes	13
My7	anti-My7	immature myeloid cells	13
My8	anti-My8	mature myeloid cells	13
My9	anti-My9	immature myeloid cells	14
NAT9	anti-NAT9	mature myeloid cells	15
HLA-DR	AAS-1	B cells, immature myeloid cells	Hokland, unpublished results

B. Perussia. The NK component was evaluated with the N901 antibody. For the evaluation of the myeloid component we used MoAbs of the My-series produced and characterized by J.D. Griffin. The My4 antigen is expressed on mature and immature monocytes as well as on acute monocytic leukemia cells; My7 is expressed preferentially on immature myeloid cells not including the CFU-C. My8 is expressed on more mature myeloid cells and to a certain degree on peripheral blood monocytes. The My9 antigen is expressed on immature myeloid cells including some CFU-C and BFU-E. In contrast, the NAT-9 antibody, which exclusively reacts with mature myeloid cells, was provided by Dr R.B. Andreasen. Finally, HLA-DR antigens were detected using the AAS-1 antibody produced in our laboratory, which reacts with a HLA-DR framework antigen. All antibodies were used as ascites in dilutions varying from 1:50 to 1:450

Indirect immunofluorescence assay Blood and bone marrow cells were adjusted to a concentration of 1 × 10^6/mL, and 0.5 to 1 × 10^6 cells were incubated for 30 minutes at 4 °C with appropriately diluted ascites containing the aforementioned antibodies. After two washes in HBSS plus 2.5% AB serum, the cells were incubated with a rabbit antimouse antiserum (Dakopatts, Copenhagen) at a dilution of 1:20 at 20 °C for 30 minutes. After two additional washes in HBSS plus 2.5% AB serum supplemented with 1% formaldehyde, the number of positive cells reactive with the different antibodies was scored by fluorescence microscopy after counting at least 200 cells.

RESULTS

Analysis of peripheral blood mononuclear cells. Many of the patients exhibited varying degrees of lymphopenia, resulting in decreased absolute numbers of most of the subsets measured. The group of patients was very heterogeneous with respect to B1 antigen expression since only nine of the patients were within normal range, eight patients exhibiting significantly higher numbers and 12 patients exhibiting lower numbers of B lymphocytes. In 23 of 29 patients the absolute numbers of B1+ cells were decreased. Using the anti-My4 antibody–defining monocytes, the same pattern as that seen for B1 was observed. However, very high numbers were seen in two patients who later progressed to chronic myelomonocytic leukemia (CMML) (data not shown).

In Fig 1 the results from the analysis of T cells and their subsets are given. Using the UCHT-1 antibody as a pan-T cell marker, the absolute numbers of cells were decreased in 24 out of 29 patients. With regard to T cell subsets, a marked heterogeneity was observed with respect to the expression of the T4 antigen, which is a reliable marker for the helper/inducer subset. However, strikingly decreased amounts of circulating T4 cells were seen in 27 out of 29 patients. In contrast, 14 of 29 patients showed increased numbers of cytotoxic/suppressor cells as evaluated by MoAb B.99.1 directed against the T8 antigen (Fig 1), with only seven exhibiting slightly decreased circulating T8 cells. These changes are reflected in the ratios between helper and suppressor cells (T4/T8 ratios), which were decreased in 17 of the 29 patients (data not shown). Moreover, four of the patients with normal or increased ratios subsequently converted during the observation period.

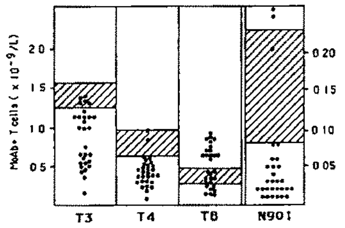

Fig 1. Reactivity of peripheral blood mononuclear cells from 29 patients using T cell-specific reagents: T3, reactivity with UCTH-1 pan-T MoAb; T4, reactivity with the B.66.6 MoAb specific for helper/inducer T lymphocytes; T8, reactivity with MoAb B.99.1 specific for cytotoxic/suppressor T cells; and N901, reactivity with the NK-specific MoAb N901. Hatched areas represent the mean and SD of 15 normal donors. All results are given as absolute numbers (× 10^{-9}/L)

Many reasons for this disturbance in T cell subsets can be envisaged, but in Fig 2 we have related the number of transfusions given to the individual patients to their T4/T8 ratio. It will be seen that with an increasing number of transfusions a steep fall in the T4/T8 ratio is seen during the first 20 transfusions, followed by a more gradual one up to 200 transfusions. Finally, with respect to the analysis of peripheral blood, we looked at the percentage of N901-bearing cells, which have been shown to contain the NK cell fraction.[12] A marked decrease was observed in 26 out of 29 patients (Fig 1), with extremely low numbers (less than 10% of that seen in normal donors) in 14 patients.

Since the aforementioned observed changes in the proportion of lymphocyte subsets could be of major importance in the etiology and pathogenesis of the anemia seen in these patients, we found it of major importance to evaluate the consistency of the observed abnormalities with respect to

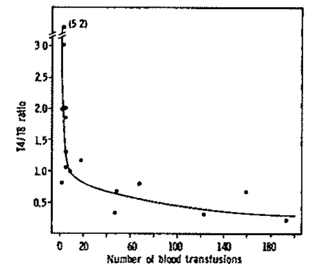

Fig 2. Correlation between T4/T8 ratios and number of blood transfusions given to 16 RA patients in whom inverted ratios had developed as of April 1985.

Table 2. Longitudinal Determinations of Leukocyte Differentiation Antigens in RA

			Antigen							
Patient	Sample	Date	MY4	MY7	MY9	B1	T3	T4	T8	N901
ER	PB	1/25/83	25*	NT	NT	7	60	17	41	17
ER	PB	3/21/83	20	NT	NT	11	63	21	39	12
ER	PB	5/26/83	17	NT	NT	9	71	25	44	15
EB	BM	11/30/82	6	11	4	4	7	NT	NT	NT
EB	BM	5/9/83	7	13	3	3	5	NT	NT	NT
EB	BM	7/12/83	4	11	4	5	10	NT	NT	NT

Abbreviations: PB, peripheral blood, NT, not tested, BM, bone marrow.

*Percentage of positive cells.

short-term reproducibility. From Table 2, which shows longitudinal measurements on peripheral blood cells from the same patient on three different occasions, it will be seen that apart from minor fluctuations, the aforementioned observed tendencies were very stable.

Analysis of mononuclear bone marrow cells. Bone marrow aspirations or Jamshidi bone marrow biopsies from the iliac crest were attempted in all patients, and suitable material was obtained in 30 out of 32 patients. In one patient, three different aspirations were performed, all of them contaminated with peripheral blood cells to such an extent that proper analysis could not be performed. In another patient, ordinary aspirations from the sternum resulted in a dry tap, and attempts to recover cells from several Jamshidi biopsies failed. The analysis of the remaining 30 patients is summarized in Figs 3 to 5; not shown are data demonstrating that the mononuclear bone marrow suspensions contained low numbers of both T and B cells as evaluated by B1 and T3 (thus excluding a gross admixture of peripheral blood in these aspirates). Cells of the myeloid lineage were evaluated using five different MoAbs, all related to different stages of myeloid differentiation. In normal humans My7 is usually expressed on less than 10% of mononuclear bone marrow cells, but it will be seen from Fig 3 that the My7 was expressed on an increased percentage of cells in 26 out of 29 patients. In contrast, the expression of My9, which is somewhat more broadly reactive, was increased in only 11 out of 29 patients. The My8 and NAT-9 antibodies react with major fractions of normal bone marrow cells, and from the data shown in Fig 4 it will be seen that between 20% and 70% of the cells from the preleukemia patients expressed these antigens, and only in a minority of cases in increased numbers. Finally, the percentages of HLA-DR–bearing cells in the bone marrows are also shown in Fig 4, and it is clear that few significant increases were observed.

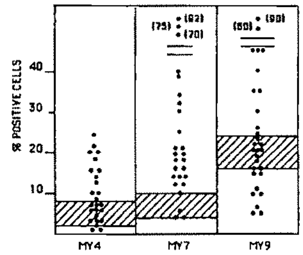

Fig 3. Distribution of monocytoid (anti-My4+) and early myeloid (anti-My7+ and anti-My9) cells in bone marrows from 30 RA patients. Hatched areas represent the mean and SD of six normal donors.

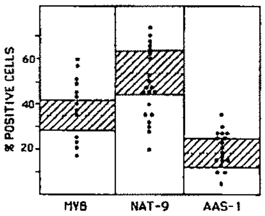

Fig 4. Reactivity of two mature myeloid differentiation antigens (My8 and NAT-9) and HLA-DR (as evaluated by MoAb AAS-1) in RA patients. The mean and SD of six normal donors are shown in the hatched areas.

To further elucidate the significance of the increased percentages of My7+ cells in the bone marrow (Fig 3), we compared them to the morphology and clinical course. Choosing the combined percentages of myeloblasts and promyelocytes as indicators of bone marrow maturity, it could be demonstrated (Fig 5) that patients with subsequent progression from RA or RA-S to RA with an excess of blasts (RAEB) exhibited high numbers of My7+ cells but usually no or modest abnormalities in morphology ($P < .01$, Wilcoxon's rank sum test). Moreover, patients with very high numbers of My7+ cells were characterized by large transfusion needs and fast disease progression either to RAEB, AML, CMML, or death resulting from severe thrombocytopenia related to bone marrow depression.

As was the case with blood specimens, short-term longitudinal studies were also performed on bone marrow cells. From Table 2 it will be seen that in a patient with slightly increased numbers of My7+ cells but stable transfusion needs the antigen expression remained stable for a 12-month period.

DISCUSSION

Until recently, the diagnostic criteria for myelodysplastic syndromes were ill defined, and consequently several groups of patients, including those with CMML, smouldering AML, or RAEB were grouped together in preleukemia patient studies. With the recent definition of diagnostic criteria,[1] a group of preleukemia patients with RA or RA-S are defined as those having less than 5% blasts in the bone marrow and

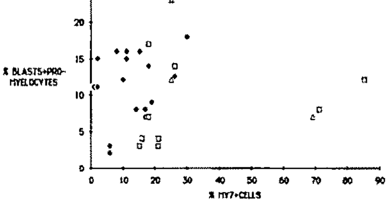

Fig 5. Correlation between My7 reactivity and morphology (combined percentages of myeloblasts and promyelocytes) in RA patient bone marrows at presentation. Key: ◊, patients remaining in RA; □, patients progressing to RAEB; Δ, patients progressing to AML (clinical staging as of April 1985).

less than 1% in peripheral blood, and in this study these diagnostic prerequisites were followed. Consequently, several patients with more than 5% blasts have been excluded from this investigation, but will be described separately.

Until now, laboratory studies in preleukemia patients have mainly consisted of cytogenetic analysis and determination of growth in semisolid agar.[2,3] These studies have shown that the majority of the patients have distinct clonal abnormalities accompanied by altered growth capacities in CFU-C assays, indicating that in at least some of the patients malignant clones are present. In contrast, few studies have dealt with detailed immunologic investigations in these patients in spite of the fact that overt AML develops in some of the patients at a later stage. This condition could thus provide a rare opportunity for studying the immune system at a potentially premalignant stage, and we therefore decided to undertake a detailed study of various aspects of the immunologic response in these patients. Here we have described the results from the analysis of blood and bone marrow mononuclear cells using a panel of MoAb suited for evaluating the major lineages of hematopoiesis.

Even though most malignant blood diseases originate in the bone marrow, evaluation of leukocyte subsets in the peripheral blood can yield valuable information concerning their possible progression and give indications on functional assays that could elucidate their pathogenesis. The most interesting finding emerged from the blood suppressor cells (Fig 1) that resulted from decreased numbers of T4 cells and increased numbers of T8 cells. Similar findings were recently published by Anderson et al in a smaller group of patients,[16] but we have extended these findings considerably by demonstrating that such patients exhibited a marked heterogeneity, with a range of T4/T8 ratio from 0.3 to 5.2. Since suppressor cells are thought to influence erythropoiesis, this heterogeneity might be of importance in determining the cause of the anemic stage in these patients, but we have at present no data suggesting that in patients presenting with low T4/T8 ratios more severe anemia develops than in those with normal ratios. Based on these findings, studies are, however, under way to analyze the influence of T4 and T8 cells (using either fluorescence-activated sorted cells or in vitro propagated T cell clones) on autologous CFU-C and CFU-E.

The anemic stage does in some patients require frequent blood transfusions, and we have—in light of the recent findings of altered T4/T8 ratios in hemophilic patients given factor VIII concentrate[17]—tried to correlate the number of transfusions given to the patients and their T4/T8 ratios. As shown in Fig 2, it appeared that patients with the highest number of blood transfusions were the ones with the lowest T4/T8 ratios. We can at present give no explanation for this, but it is tempting to suggest that immunosuppressive agents (microbiologic or nonmicrobiologic) are transferred during blood transfusions. Whether this (or these) agent is related to the ones purportedly demonstrated in patients with acquired immunodeficiencies[18] remains to be established. Another possibility could be that these changes are caused by the transfer of cytomegalovirus (CMV) with the tranfusions, but since these were not screened for CMV, we have not been able to address this question.

To a certain degree, our findings contrast to those of Anderson et al who found a mean T4/T8 ratio of 1.8.[16] However, as previously mentioned, many of these patients do not fit into the more strict criteria for primary preleukemia,[1] and these studies are therefore not readily comparable with ours.

When evaluating the T cell component using pan-T MoAb (Fig 1), an increased percentage of T cells was demonstrated in some of the patients, who also exhibited lowered percentages of B cells, as evaluated by the MoAb anti-B1. These data could suggest that the increased numbers of T8+ lymphocytes exert suppression on the B lymphocyte lineage, but final proof of this awaits functional studies of the in vitro immunoglobulin secretion in these patients, and such are now in progress. It is, however, interesting to note that Anderson et al[16] using anti-immunoglobulin antisera in three preleukemic patients did not find a similar decrease in the number of B cells. These authors did, however, observe a lack of Epstein-Barr virus receptors on B lymphocytes in their patients, and based on this finding it was hypothesized that the B cells in the preleukemia patients might have a deficiency in mature immunocompetent B cells.

The natural history of the development in the single patient from a protracted stage of RA through RAEB to AML is only partly elucidated, and obviously disease parameters of predictive value regarding malignant transformation are highly sought after since these patients are difficult to treat once they have reached the AML stage. With this in mind, the finding of greatest potential interest that emerged from our study is the increased numbers of immature myeloid cells in the bone marrows that reacted with the antibodies anti-My7 and to a lesser extent anti-My9 since this adds considerably to our knowledge of the use of antimyeloid MoAbs in myeloid differentiation disorders. This finding is best seen in light of our comparative studies (Fig 5) that clearly demonstrated that progression from the initial RA stage to RAEB could not be predicted by bone marrow morphology. In contrast, patients with high numbers of My7+ cells (irrespective of morphology) seemed to be the ones susceptible to RAEB progression. In connection with this, it should be noted that progression to RAEB was always accompanied by an increase in My7+ cells in the bone marrow. However, the My7 determinations shown in Fig 5 are all from the time of diagnosis.

The immunologic heterogeneity observed in our patients might be useful in the longitudinal studies performed for the purpose of prognostication and demonstration of changes indicating a transformation to an overt AML. Regarding this, it is noteworthy that during a short observation period (less than 6 months) the antigen expression remained stable, thus demonstrating the validity of these assays (Table 2). The apparent asynchrony between My7 expression and morphology thus indicates that immunologic phenotyping in RA patients should be included in the diagnostic armature. Naturally, My7 is of primary importance in this context, but it might well be envisaged that an immaturity index of a given bone marrow might be constructed that could yield even more information. This might—apart from My7—include HLA-DR antigens that are known to be present on large proportions of AML cells as well as a mature myeloid antigen, eg, NAT9, that, in contrast to My8, is not expressed by monocytes. Consequently, the combined presence of My7 and HLA-DR (AAS-1) and a decrease in NAT-9 expression might be more predictive than My7 alone. Construction of such ratios might possibly also be of importance in monitoring the response to treatment of AML patients.

Our understanding of the immune response against early neoplasias has been drastically altered during the recent years. Earlier assumptions that educated cytotoxic T lymphocytes might kill emerging malignant clones have not been fulfilled, and it is now believed that the NK cell is a more likely candidate for cell-mediated lysis of malignant cells. We have shown earlier that preleukemic patients are deficient in NK function,[4] and here we extend this conclusion by demonstrating decreased numbers of cells reactive with the recently described NK-specific monoclonal antibody N901. This finding strongly indicates that the reason for the deficient NK activity in these patients is a lack of circulating NK cells. Whether this is due to altered migratory capacities of NK cells in these patients (possibly involving selective localization close to abnormal clones of premalignant myeloid cells) is not clear, but estimation of NK activity and N901+ cells from other tissues is under way to resolve the issue. Preliminary results from such experiments do in fact suggest that inhibition of CFU-GM growth can only be obtained after preincubation with peripheral blood mononuclear cells with high NK activity (as measured in a chromium release assay).

In addition to NK cells, T8-positive T lymphocytes might be envisaged to inhibit myeloid growth through the secretion of suppressive factors. The finding of increased proportions of T8-positive suppressor cells in the blood of all patients might support this, but the final answer for this question awaits either semisolid agar experiments or immunoperoxidase staining of bone marrow sections with relevant MoAbs.

In conclusion, we have demonstrated a number of abnormalities in the distribution of leukocyte subsets from blood and bone marrow in primary preleukemic patients. Of these, the decreased T4/T8 ratio in the blood and the increased proportion of cells in the bone marrow possessing the immature myeloid antigen My7 might be of importance in the pathogenesis and for the diagnosis of this potentially premalignant disease.

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Deficient Induction of Leukotriene Synthesis in Human Neutrophils by Lipoxygenase-Deficient Platelets

By Kenji Kanaji, Minoru Okuma, and Haruto Uchino

The effect of human platelets with deficient lipoxygenase activities on leukotriene B_4 (LTB_4) synthesis by neutrophils was studied. When arachidonic acid (AA) metabolites obtained from the incubation of washed normal neutrophils and platelets with *N*-formylmethionylleucylphenylalanine (FMLP), cytochalasin B, and AA were analyzed by reversed-phase high-perfomance liquid chromatography, the synthesis of 5-lipoxygenase products, including LTB_4, was remarkably stimulated by platelets, with their maximal effect at a ratio of platelets to neutrophils of 15:1. However, the use of lipoxygenase-deficient platelets obtained from four patients with myeloproliferative disorders instead of normal platelets showed the deficient production of 5-lipoxygenase–derived products, whereas platelets with normal lipoxygenase activities obtained from MPD patients stimulated the 5-lipoxygenase pathway similarly to the way in which normal platelets did. The addition of 12-hydroperoxyeicosatetraenoic acid (12-HPETE), a labile AA metabolite via the platelet lipoxygenase pathway, could activate the 5-lipoxygenase pathway in neutrophils incubated with FMLP, cytochalasin B and AA, but its stable end product, 12-hydroxyeicosatetraenoic acid, could not. Thus, it is suggested that lipoxygenase-deficient platelets did not sufficiently stimulate LTB_4 synthesis during platelet-neutrophil interactions because of defective formation of 12-HPETE. This altered interaction between platelets and neutrophils through the lipoxygenase pathway might result in deficient responses at sites of thrombosis or inflammation in patients with deficient platelet lipoxygenase activities.

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ARACHIDONIC ACID (AA) is converted via the 5-lipoxygenase pathway to eicosanoids, including leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE), in polymorphonuclear neutrophils. LTB_4 has potent chemotactic and chemokinetic activities and may be one of the major mediators of neutrophil function.[1] On the other hand, platelets contain 12-lipoxygenase as well as cyclooxygenase. The former enzyme converts AA to 12-hydroperoxyeicosatetraenoic acid (12-HPETE), which is reduced by peroxidase to 12-hydroxyeicosatetraenoic acid (12-HETE).[2] Although the significance of the platelet 12-lipoxygenase pathway in platelet function is still obscure, recent studies have demonstrated the presence of biochemical interactions between platelets and neutrophils via lipoxygenase enzymes. Borgeat et al have shown that 5S,12S-dihydroxyeicosatetraenoic acid (5S,12S-diHETE) is a product of the successive reactions of AA with 5-lipoxygenase and 12-lipoxygenase.[3,4] Marcus et al have demonstrated that AA and 12-HETE derived from platelets serve as substrates for eicosanoid formation by neutrophils[5] and that both neutrophils and platelets need to be activated by a strong stimulus such as calcium ionophore A23187 for the production of significant amounts of 5S,12S-diHETE.[5,6] Furthermore, Maclouf et al have reported that platelet-derived 12-HPETE stimulates LTB_4 synthesis in human blood leukocytes,[7] a finding that remains to be confirmed by other laboratories.[8]

Patients with myeloproliferative disorders (MPD) are known to have a high incidence of infections,[9] probably because of abnormal leukocyte function. Since LTB_4 may regulate neutrophil function,[10] it might be assumed that LTB_4 synthesis is decreased in these patients. The deficient production of LTB_4 may result not only from abnormalities of neutrophils themselves[11] but also from abnormalities of platelets because platelets in some MPD patients are known to be deficient in 12-lipoxygenase activity.[12,13]

In this study, we investigated the effects of platelets on AA metabolism by neutrophils, especially concerning LTB_4 synthesis, when stimulated with *N*-formylmethionylleucylphenylalanine (FMLP) and AA in the presence of cytochalasin B. Lipoxygenase-deficient platelets from MPD patients show a decreased activation of LTB_4 synthesis in neutrophils, possibly because of deficient production of 12-HPETE by the platelets.

MATERIALS AND METHODS

Patients and normal controls. Eight patients with MPD were investigated: three with chronic myeloid leukemia (CML) in the stable phase, two with polycythemia vera (PV), and three with essential thrombocythemia (ET) Diagnosis was determined by standard methods as described previously.[12] Platelet AA metabolism was estimated by the thiobarbituric acid method[14] by which deficient activities of the platelet lipoxygenase pathway were demonstrated in four of the eight patients. Seven normal volunteers were also studied as controls. Informed consent was obtained from all subjects.

Preparation of cell suspensions. Blood from normal donors and patients who had taken no drugs known to affect AA metabolism for at least 2 weeks before venipuncture was collected in 3.8% trisodium citrate solution (9 vol blood to 1 vol citrate) and centrifuged for the preparation of platelet-rich plasma as described previously.[15] Neutrophils were prepared as described previously[11] and suspended in the incubation buffer (pH 7.4) containing 50 mmol/L Tris-HCl, 101 mmol/L NaCl, and 5 mmol/L glucose. Neutrophil preparations from normal donors always contained more than 95% segmented neutrophils, and percentages of contaminating platelets were less than 30. Platelets were washed as described previously[12] and finally suspended in the incubation buffer. Contamination of the platelet suspension by erythrocytes and leukocytes was negligible.

Preparation of 12-HPETE and 12-HETE. 12-HPETE and 12-HETE were prepared by a modification of the procedure reported by Pace-Asciak et al.[16] In short, washed human platelets

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were resuspended in 50 mmol/L Tris-buffered saline, pH 7.4 (0.8 × 10^9 platelets/mL), and disrupted sonically.[17] After the lysate (8 mL) was incubated with 200 μmol/L AA in the presence of 100 μmol/L indomethacin for five minutes at 37 °C, the incubation mixture was placed on ice, immediately followed by the addition of ice-cold methanol (8 mL) and ice-cold diethyl ether (24 mL), and the mixture was acidified to pH 3.0 with 0.2 mol/L HCl. The ether phase was separated by rapid centrifugation at 4 °C and washed with 3 mL of ice-cold water. The ether phase was finally dried over anhydrous sodium sulfate and evaporated to dryness under nitrogen. The residue was dissolved in hexane and purified by high-performance liquid chromatography (HPLC) using a column of Nucleosil 50-5 (4.6 × 250 mm, Macherey-Nagel, Düren, Germany) and a mobile phase consisting of hexane/isopropanol/acetic acid (98.2:0.1, vol/vol) at a flow rate of 1 mL/min. An Altex model 110A single-pump chromatograph (Altex Scientific, Berkeley, Calif) with a Soma S-310A variable-wavelength spectrophotometer detector (Soma Optics, Tokyo) was used The identity of 12-HETE was confirmed by gas chromatography–mass spectrometry,[11] and that of 12-HPETE was confirmed by reductive conversion to 12-HETE with stannous chloride.[12] Quantitation was done by using UV absorption at 237 nm in ethanol.[18] The purified material was dissolved in acetone, stored under nitrogen at −70 °C, and used within 2 weeks The products used in the experiments were more than 97% pure, based on the HPLC analysis.

Incubation and extraction Unless otherwise specified, after cell suspensions containing 3 × 10^7 neutrophils and 0 to 75 × 10^7 (routinely 45 × 10^7) platelets/mL were incubated with cytochalasin B (5 μg/mL) for five minutes at 37 °C, the mixtures were incubated with 10 μmol/L FMLP and 60 μmol/L AA for another five minutes at the same temperature In experiments with 12-HPETE and 12-HETE, each was transferred to an assay tube, and the solvent was evaporated under nitrogen Neutrophil suspensions (3 × 10^7 cells/mL) preincubated with cytochalasin B (5 μg/mL) for five minutes at 37 °C were transferred to the assay tube without delay, immediately followed by the addition of 10 μmol/L FMLP and 60 μmol/L AA, and then the mixtures were incubated for another five minutes at the same temperature. When the calcium ionophore was used, neutrophils (3 × 10^7 cells/mL) were incubated with 10 μmol/L A23187 and 60 μmol/L AA for five minutes at 37 °C. In all experiments with cell suspensions, 1 mmol/L $CaCl_2$ was added just before the incubation at 37 °C. The incubation was terminated by the addition of 4 vol ethyl acetate after acidification by 0.2 mol/L HCl to pH 3.0, and 450 ng of prostaglandin B_2 (PGB_2) was added as an internal standard. Extraction and evaporation were performed as described previously[11] and evaporated residue dissolved in 30 μL methanol was subjected to chromatography as follows.

FMLP dissolved in the incubation buffer (1 mmol/L) and cytochalasin B in dimethyl sulfoxide (2 mg/mL) were stored at −20 °C and diluted with the incubation buffer when used. AA and A23187 were dissolved as described previously.[14,19] Dimethyl sulfoxide was at a final concentration of less than 0.25%.

HPLC analysis. Reversed-phase HPLC was routinely carried out using a column of Nucleosil $_7C_{18}$ (4.6 × 250 mm, Macherey-Nagel). The chromatograph and detector were the same as those used for preparation of 12-HPETE. A 10-μL aliquot of each sample was analyzed by the use of a mobile phase I consisting of methanol/water/acetic acid (75:25:0.01, vol/vol) at a flow rate of 1 mL/min.[20] Another 10-μL aliquot was analyzed by a mobile phase II consisting of tetrahydrofuran/methanol/water/acetic acid (25:30 45:0.1, vol/vol) at a flow rate of 0.9 mL/min for the resolution of LTB_4 and 5S,12S-diHETE.[21] Column effluent was monitored at 270 nm for PGB_2, LTB_4, and 5S,12S-diHETE and at 235 nm for 12-hydroxyheptadecatrienoic acid (HHT), 12-HETE, and 5-HETE. The amounts of 5-lipoxygenase–derived products were quantitated by comparing their peak areas with that of the internal standard (PGB_2). Th extinction coefficients used were determined in each mobile phase using a Hitachi Model 200-20 spectrophotometer (Hitachi, Tokyo) 48,700 for LTB_4 in the mobile phase (II), 27,900 for 5-HETE in the mobile phase (I), and 23,100 and 22,400 for PGB_2 in the mobile phase (I) and (II), respectively. The extinction coefficient of 5S,12S-diHETE was assumed to be identical with that of LTB_4. Identification of LTB_4, 5-HETE and 12-HETE was performed by gas chromatography–mass spectrometry.[11] 5S,12S-diHETE and HHT were identified by comparisons of the chromatographic behaviors of each authentic sample.

Materials. FMLP and cytochalasin B were obtained from Sigma Chemical Co, St Louis; authentic 5S,12S-diHETE was kindly donated by Dr Shozo Yamamoto, Department of Biochemistry, Tokushima University School of Medicine, Tokushima, Japan Authentic HHT was prepared biosynthetically as previously described.[12] The other agents were the same as described previously.[11]

RESULTS

The incubation of neutrophils with FMLP, cytochalasin B, and AA led to the formation of small amounts of 5-lipoxygenase products, including LTB_4, 5S,12S-diHETE, and 5-HETE, as illustrated in Fig 1A and 1C. However, the production of 5-lipoxygenase metabolites, especially LTB_4, was remarkably activated in the presence of platelets as shown in Figs 1B and 1D. Platelets (45 × 10^7 cells/mL) incubated under these conditions in the absence of neutrophils produced significant amounts of 12-HETE and HHT, but no detectable amounts of 5-lipoxygenase products were obtained (data not shown)

The stimulating effect of added platelets on AA metabo-

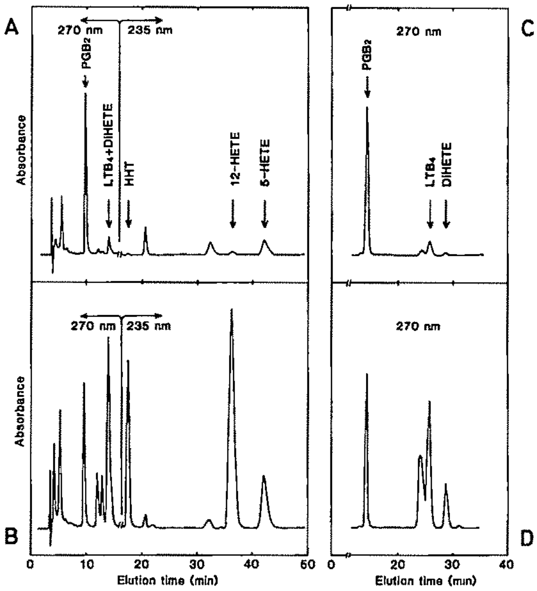

Fig 1. Reversed-phase HPLC chromatograms of AA metabolites obtained from the incubation of neutrophils with FMLP, cytochalasin B, and AA in the presence (B and D) or absence (A and C) of platelets (45 × 10^7 cells/mL). Conditions for incubation and extraction were described in Materials and Methods. The extracted products were analyzed by reversed-phase HPLC using mobile phase I (A and B) and mobile phase II (C and D) as described in Materials and Methods.

lism by platelet-neutrophil interactions depended on the ratio of platelets to neutrophils (Table 1). The production of LTB_4 and 5-HETE was increased with this ratio up to 15:1, but a further increase to 25:1 reduced the production of these metabolites. 5S,12S-diHETE generation peaked at a platelet:neutrophil ratio of 15:1. When the mixed cell suspension with the ratio of 15 to 1 was incubated with FMLP, cytochalasin B, and AA, the amount of LTB_4 produced was similar to that obtained from the incubation of neutrophils with calcium ionophore A23187 and AA (Table 1).

In experiments using mixed cell suspensions incubated with cytochalasin B and AA in the presence of varying concentrations of FMLP, the amount of LTB_4 produced was linearly increased with increasing FMLP concentrations up to 10 μmol/L, but the synthesis of 5-HETE and 5S,12S-diHETE leveled off at its lower concentration (0.1 μmol/L) (Fig 2A). In experiments with similar cell suspensions incubated with FMLP and AA in the presence of varying concentrations of cytochalasin B, 1 μg/mL or higher concentrations of cytochalasin B significantly increased LTB_4 synthesis but reduced 5S,12S-diHETE formation. No significant changes were observed in the 5-HETE formation (Fig 2B). In experiments with similar cell suspensions incubated with cytochalasin B and FMLP in the presence of various AA concentrations, the formation of LTB_4 was greatest at AA concentrations from 60 μmol/L to 120 μmol/L, 5-HETE synthesis was greatest at AA concentrations higher than 90 μmol/L, but 5S,12S-diHETE formation leveled off at 30 μmol/L AA. No 5-lipoxygenase products were consistently detectable without the addition of AA (Fig 2C).

When mixed cell suspensions were incubated with FMLP, cytochalasin B, and AA for varying periods of time, 5-HETE and LTB_4 synthesis reached a maximum within three minutes and five minutes of incubation times, respectively, and decreased thereafter, whereas 5S, 12S-diHETE synthesis reached a steady level at one- to three-minute incubations and did not decrease thereafter (data not shown).

Fig 3 shows the effect of the addition of 12-HPETE instead of platelets on the production of 5-lipoxygenase metabolites by neutrophils incubated with FMLP and AA in the presence of cytochalasin B. This unstable product from platelet lipoxygenase stimulated the production of these metabolites in a concentration-dependent manner, and the effect of 10 μmol/L 12-HPETE was comparable to that obtained when platelets and neutrophils were incubated with FMLP, cytochalasin B, and AA. In contrast, stimulation was not observed when 12-HETE, a stable end product of the platelet lipoxygenase pathway, was added to the incubation mixture at the same concentrations (up to 10 μmol/L, data not shown).

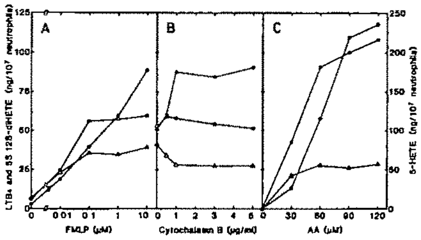

Fig 2. Effects of stimulant concentrations on the synthesis of 5-lipoxygenase products by platelet-neutrophil interactions. Cell suspensions containing neutrophils and platelets (45 × 10^7/mL) were incubated with varying concentrations of FMLP (A), cytochalasin B (B), or AA (C) as indicated in the presence of fixed concentrations of other two stimulants. The concentrations of FMLP, cytochalasin B, and AA were 10 μmol/L, 5 μg/mL, and 60 μmol/L, respectively. Products were analyzed as described in Materials and Methods. (A) and (B) were representatives of two experiments that yielded similar results, and (C) was that of three such experiments: Key: ●—●, LTB_4; ○—○, 5-HETE; △—△, 5S,12S-diHETE.

Table 2 shows the effects of platelets from MPD patients on the production of 5-lipoxygenase metabolites by neutrophils from normal donors. Lipoxygenase-deficient platelets from four patients including two each with CML and ET (patients 1 to 4 in Table 2) brought about significantly reduced production of 5-lipoxygenase metabolities by neutrophils compared with normal platelets ($P < .001$, $P < .001$, and $P < .01$ for LTB_4, 5S,12S-diHETE, and 5-HETE, respectively), whereas platelets with normal lipoxygenase activities from four patients including two with PV and one each with ET and CML (patients 5 to 8 in Table 2) activated 5-lipoxygenase similarly to platelets from normal donors. Furthermore, it was demonstrated that the 5-lipoxygenase pathway in neutrophils from a patient with platelet lipoxygenase deficiency (patient 4 in Table 2) was normally stimulated by platelets from a normal donor, although

Table 1. Effects of Platelet Numbers Added (Experiment I) and A23187 (Experiment II) on the Synthesis of 5-Lipoxygenase Metabolites by Neutrophils

	Platelet/ Neutrophil Ratio	LTB_4*	5S,12S-diHETE*	5-HETE*
Experiment I†	0	15.5 ± 4.9	1.3 ± 0 8	38.5 ± 14.7
	5	62.7 ± 30.9	6.9 ± 2.9	80.0 ± 5.3
	15	105.2 ± 2.2	33 3 ± 9 0	125.2 ± 19.3
	25	79.4 ± 7.0	45 1 ± 17.8	79.0 ± 16 7
Experiment II‡	0	106.8 ± 7.0	17.6 ± 1.9	432.9 ± 27.5

*ng/10^7 neutrophils.

†Neutrophils, cytochalasin B, AA, and FMLP were incubated as described in Materials and Methods in the presence or absence of varying numbers of platelets as indicated.

‡Neutrophils were incubated with A23187 and AA as described in Materials and Methods.

Mean values ± SD of three experiments.

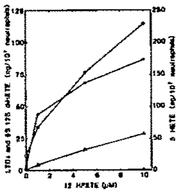

Fig 3. Effects of 12-HPETE on the synthesis of 5-lipoxygenase products by neutrophils. The condition of incubation, isolation of lipid products, and method of HPLC analysis were described in Materials and Methods. The data were representative of three separate experiments that gave similar results. Symbols are the same as those in Fig 2.

the stimulation by this patient's platelets was reduced (Table 3).

DISCUSSION

Our experiments demonstrate that the synthesis of 5-lipoxygenase metabolites, including LTB_4, 5-HETE, and 5S,12S-diHETE, by human neutrophils was enhanced by their incubation with human platelets in the presence of FMLP, cytochalasin B, and AA and that the effect of lipoxygenase-deficient platelets on AA metabolism was significantly decreased. FMLP is a formylated peptide similar to bacterial chemotaxins and activates various neutrophil functions.[22,23] Stimulation of human neutrophils with the peptide in the presence or absence of cytochalasin B was reported to form a small amount of LTB_4[24,25] and its ω-carboxyl derivative.[26] In our experimental conditions, FMLP was considered an effective stimulant of 5-lipoxygenase metabolism since the amount of LTB_4 increased markedly with increasing FMLP concentrations up to 10 μmol/L (Fig 2A). Cytochalasin B was not essential to the consistent production of LTB_4 but augmented the effect of FMLP at concentrations of 1 to 5 μg/mL (Fig 2B) The addition of AA to the incubation mixture appeared to be essential to the activation of the 5-lipoxygenase pathway because the platelet-neutrophil mixture incubated with FMLP alone in the presence of cytochalasin B produced no detectable amount of the 5-lipoxygenase products (Fig 2C). Furthermore, there existed a critical ratio of platelets to neutrophils (15:1) in the presence of exogenous AA for the maximal LTB_4 production (Table 1).

Table 3. Effects of Normal or Lipoxygenase-Deficient Platelets on the 5-Lipoxygenase Metabolism in Neutrophils From a Patient With the Lipoxygenase-Deficient Platelets

Platelets	Platelet/ Neutrophil* Ratio	LTB_4†	5S,12S-diHETE†	5-HETE†
None	0	3.1	0	9.5
Patient's	5	19.0	1 0	33.0
Patient's	15	30.6	1.8	40.7
Normal	5	35.3	3 2	56.4
Normal	15	71.5	20.1	99 0

*Neutrophil suspension obtained from patient 4 in Table 1 contained 67% segmented neutrophils, 18% band neutrophils, 13% neutrophilic myelocytes and metamyelocytes, and 2% eosinophils.

†ng/10^7 neutrophils.

Since the 5-lipoxygenase activity has not been demonstrated in human platelets,[2,18,27] this increase in 5-lipoxygenase metabolites by platelet-neutrophil interactions was not considered a result of the conversion by the platelets themselves of AA to 5-HPETE, an unstable intermediate of the 5-lipoxygenase pathway.[1] Consumption of AA in the cell suspension medium by platelets via cyclo-oxygenase and 12-lipoxygenase pathways and liberation of AA from platelet phospholipids into the medium could occur, and they might alter the amount of this fatty acid available to neutrophils. Furthermore, platelet-derived AA has been reported to be transformed to LTB_4 and 5-HETE by stimulated neutrophils.[5] However, these possible alterations in the AA concentration in the medium could not account for the stimulating effect of platelets on the LTB_4 production since LTB_4 produced by the incubation of neutrophils with the stimulants used in our experimental system was not significantly affected by the addition of AA from 30 μmol/L to 120 μmol/L (data not shown). In our incubations, time-course studies that demonstrated the transient increase of LTB_4 lessened the possibility that platelets reduced the transformation of LTB_4 via the ω-oxidation pathway in neutrophils[26,28,29] so that LTB_4 might accumulate in the incubation mixture with a five-minute incubation. Therefore, it was

Table 2. Effects of Platelets From Patients With MPD on the Synthesis of 5-Lipoxygenase Products by Normal Neutrophils

Patient No	Age (yr)	Sex	Diagnosis	PLO*	LTB_4†	5S,12S-diHETE†	5-HETE†
1	51	F	ET	0.29	51.2	4.4	35.8
2	27	F	ET	0.05	29.8	3.1	67.8
3	54	M	CML	0.10	56.9	2.6	60.2
4	48	F	CML	0.06	52 7	2.2	40.0
5	63	F	PV	1.18	88.8	30 7	111.4
6‡	55	M	PV	0.73	108 4	28.6	88.7
7	36	F	ET	1 00	104 9	23.6	124.6
8	45	M	CML	0 74	88 8	27.1	90.6
Normal ranges (mean ± 2 SD)				1.17 ± 0.68	101 9 ± 20 0	30.6 ± 17 5	133.6 ± 78.1
(n)				(31)	(7)	(7)	(7)

Abbreviation: PLO, platelet lipoxygenase.

*nmol malondialdehyde/10^8 platelets.[14]

†ng/10^7 neutrophils.

‡Platelets of this patient showed a subnormal response to thromboxane A_2 similar to our previous patient[19] (data not shown)

suggested that some platelet-derived factors other than AA per se contributed to activate the 5-lipoxygenase pathway of neutrophils.

Adenosine triphosphate and adenosine diphosphate, major constituents of platelet-dense granules, were demonstrated to activate the 5-lipoxygenase enzyme isolated from guinea pig peritoneal polymorphonuclear leukocytes.[30] However, these granule contents seemed to be of minor importance for the platelet activation of LTB_4 production by neutrophils since supernatant from washed platelet suspension in which the release reaction had been completed by thrombin showed no significant effect on the production of 5-lipoxygenase metabolities (data not shown), although the supernatant might induce production of 12S,20-dihydroxyeicosatetraenoic acid.[6] Furthermore, the use of platelets with defective release mechanisms and with normal AA metabolism prepared from patient 6 in Table 2 showed normal stimulating effects on the 5-lipoxygenase metabolism (Table 2).

Since the consistent production of 5-lipoxygenase metabolites required exogenous AA (Fig 2C), it was suggested that AA was necessary as a substrate of the 5-lipoxygenase enzyme as well as for the conversion to a metabolite with the stimulating activity by platelets. However, metabolites via the platelet cyclo-oxygenase pathway seemed to be of little significance for the platelet-induced stimulation because the addition of 500 μmol/L aspirin to the incubation mixture did not reduce the production of 5-lipoxygenase metabolites (data not shown).

The addition of 1 to 10 μmol/L 12-HPETE to neutrophils incubated with FMLP, cytochalasin B and AA stimulated the synthesis of LTB_4, 5-HETE, and 5S,12S-diHETE, whereas its stable derivative, 12-HETE, had no stimulating effect. Similar data were reported by Maclouf et al,[7] although we did not see whether 12-HPETE alone could stimulate LTB_4 production under their experimental conditions, which were different from ours. These results demonstrate an important role of 12-HPETE for the platelet-induced activation of the 5-lipoxygenase pathway during platelet-neutrophil interactions, but the activation mechanism remains to be elucidated.

Stimulating effects of platelets on AA metabolism by neutrophils were decreased when lipoxygenase-deficient platelets from MPD patients (patients 1 to 4 in Table 2) were mixed with neutrophils from normal donors. In contrast, platelets with normal lipoxygenase activities from MPD patients (patients 5 to 8 in Table 2) were no different than those of normal donors in their stimulating effect on LTB_4 synthesis during platelet-neutrophil interactions. In the case of a patient with lipoxygenase-deficient platelets (patient 4 in Table 2), the patient's neutrophils were normally activated by platelets from a normal donor and not by the patient's own platelets (Table 3); in patients with normal activities of the platelet lipoxygenase pathway, AA metabolism of the patients' neutrophils were stimulated by their own platelets in a fashion similar to that in the experiments using normal donors' platelets (data not shown). These findings also confirmed the role of 12-HPETE for the effect of platelets. Since even the lipoxygenase-deficient platelets stimulated the 5-lipoxygenase metabolism to some extent, potentiation of this metabolism by platelets may proceed through various mechanisms, only some of which involve 12-HPETE, which was studied here.

It has been suggested that LTB_4 synthesis plays an important role for some functions of neutrophils, including adhesion, chemotaxis, aggregation, lysosomal enzyme release, and generation of superoxide.[1] Therefore, our results suggest that platelets may contribute to both potentiation of inflammatory reactions and the recruitment of neutrophils to vascular lesions. Thus, synthesis of LTB_4 may play a role in thrombosis and hemostasis. It is also possible that the deficient stimulation of LTB_4 synthesis by lipoxygenase-deficient platelets from MPD patients may contribute to the high incidence of infections and vascular complications of these patients.[9]

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Chemoattractant-Mediated Stimulation of the Respiratory Burst in Human Polymorphonuclear Leukocytes May Require Appearance of Protein Kinase Activity in the Cells' Particulate Fraction

By Marilyn C. Pike, Laszlo Jakoi, Linda C. McPhail, and Ralph Snyderman

Low doses of aliphatic alcohols produce divergent effects on the function of chemoattractant receptors on human polymorphonuclear leukocytes (PMNs) since they enhance chemotaxis but inhibit stimulation of superoxide production by chemoattractants. As such, alcohols can provide useful pharmacologic tools to probe the mechanisms of stimulus-response coupling in leukocytes. A role for protein kinase C has been implicated in the activation of the respiratory burst in PMNs. Although the vast majority of this enzyme activity is located in the cytosolic fraction of unactivated PMNs, protein kinase C activity appears in the particulate fraction of the cells when they are stimulated to produce superoxide by either chemoattractants or by phorbol myristate acetate (PMA). Doses of the alcohols that selectively inhibited stimulation of superoxide production by chemoattractants also inhibited the appearance of protein kinase C activity as well as an undefined protein kinase activity in the particulate fraction of the cells. In contrast, the alcohols did not affect either the ability of PMA to stimulate the production of superoxide in PMNs nor the appearance of protein kinase activity in the cells' particulate fraction. PMA is known to bind and activate protein kinase C directly, thus bypassing receptor-mediated events. These data suggest that alcohols inhibit the stimulation of the respiratory burst by chemoattractants in PMNs by blocking the ability of receptor occupancy to induce the appearance of protein kinase activity in particulate fractions. These results moreover suggest that the appearance of protein kinase activity in the particulate fraction may be required for activation of the respiratory burst in PMNs.

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CHEMOATTRACTANT receptor occupancy in leukocytes results in a variety of functional responses including morphologic polarization, chemotaxis, secretion of lysosomal enzymes, and activation of the respiratory burst.[1,2] Several lines of evidence suggest that motility-related functions are regulated quite differently from secretion and superoxide production.[2,3] The concentrations of chemoattractants required to initiate the former are about 20-fold less than those required to stimulate the latter.[2,4,5] In addition, low doses of aliphatic alcohols increased the affinity of the oligopeptide chemoattractant receptor, enhanced PMN chemotaxis, yet selectively depressed chemoattractant-mediated superoxide production and lysosomal enzyme secretion.[6] The alcohols appeared to alter stimulus-response coupling by the chemoattractant receptor since superoxide release induced by phorbol myristate acetate (PMA) was unaffected.[6] Recent evidence suggests a role for protein kinase C (PKC) in superoxide production by PMNs, human monocytes, and mouse macrophage cell lines stimulated by PMA or oleoyl-acetyl glycerol.[7-10] PKC activity in unstimulated human PMNs was almost entirely cytosolic when the cells were disrupted in the presence of ethylene glycol tetra-acetic acid (EGTA), but chemoattractants and PMA induced the appearance of PKC activity in the particulate fractions.[9,11] The induction of PKC activity in the particulate fraction by chemoattractants was correlated with activation of the respiratory burst rather than with motility-related functions in that it occurred at high doses of chemoattractant and was greatly enhanced by cytochalasin B.[11] In the present work, we have used low doses of alcohols as a pharmacologic probe to determine whether superoxide release stimulated by the oligopeptide chemoattractant receptor requires the appearance of PKC activity in the particulate fractions of PMNs.

MATERIALS AND METHODS

PMA, phosphatidylserine (PS), 1,2-diolein, histone (type III), cytochrome c, cytochalasin B, f-Met-Leu-Phe (FMLP) and adenosine triphosphate (ATP) were from Sigma Chemical Co (St Louis). Gamma[^{32}P]ATP was from New England Nuclear (Boston). Trifluoperazine (TFP) and purified calmodulin were generously supplied by Drs Marty Vogel and David Sedwick, Duke University Medical Center

Cell preparation and isolation of subcellular fractions. Human blood PMNs were isolated as previously described.[12] Cells (1×10^7 in 5.0 mL) were preincubated in Hanks' balanced salt solution (HBSS) containing 10 mmol/L HEPES and 4.2 mmol/L $NaHCO_3$ (GIBCO, Grand Island, NY), pH 7.5, at 37 °C for five minutes with 10 μmol/L cytochalasin B with or without alcohols FMLP (100 nmol/L) was added for one minute or PMA (1 ng/mL to 1 μg/mL) was added for five minutes Reactions were stopped by addition of a tenfold excess of ice-cold, calcium-free HBSS containing the appropriate concentration of alcohol Cells were centrifuged, washed with alcohol-free HBSS, and the cell pellets were suspended in extraction buffer consisting of 0.05 mol/L Tris-HCL, 0 05 mol/L 2-mercaptoethanol (2-ME), 2 mmol/L EGTA, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), pH 7 5,[13] and sonicated for 30 seconds at a setting of 35% using an Artek Sonic 300 Dismembranator equipped with a microtip (Artek Systems Corp, Farmingdale, NY). Following centrifugation for one hour at 100,000 g, the supernatant (cytosol) was removed and the pellet extracted with 1.0 mL of 1% Triton X-100 (Sigma) in extraction buffer. Following centrifugation for one hour at 100,000 g, the supernatant (extractable particulate fraction) was removed and the nonextractable pellet resuspended in 1.0% Triton X-100 in extraction buffer.

Assay of protein kinase C. Reaction mixtures contained 0.03 mol/L Tris-HCL, pH 7 5, 0.01 mol/L $MgCl_2$, 50 μmol/L ATP, 2×10^6 cpm [γ^{32}P]ATP, 0.4 mmol/L EGTA, 0.01 mol/L 2-ME and 0.2

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mmol/L PMSF. Where indicated, 5 μg of PS, 0.5 mg of diolein, and 0.6 mmol/L $CaCl_2$ were included.[13,14] Reactions were started by adding 0.05 mL of enzyme preparation in extraction buffer containing 0.05% Triton X-100 (final reaction volume 0.25 mL) and incubated at 30 °C for five minutes for cytosolic fractions or 60 minutes for particulate fractions. The reactions were terminated with 1 mL of 25% trichloroacetic acid and 50 μg of bovine serum albumin, filtered, and counted.[14] PKC activity in cytosolic and Triton-extractable particulate fractions is expressed as pmol ^{32}P incorporated/min/10^7 cells and is given as the difference between the activity in the presence of PS, diolein, and calcium and that in the absence of these agents. A protein kinase activity present in the nonextractable particulate fraction was assayed as stated except that the PS, diolein, and calcium were not added since these substances did not affect the activity of this kinase. The concentrations of alcohols used in these studies did not affect any kinase activity when added directly to the enzyme assays (data not shown).

Assay of superoxide. Superoxide release by human neutrophils was assayed by monitoring the superoxide dismutase–inhibitable reduction of ferricytochrome c using an extinction coefficient of 29.5/mmol/L/cm as described[6] except that reaction mixtures contained 2.5 × 10^6 cells.

Determination of microviscosity parameter. Microviscosity was quantified by measuring the fluorescence polarization of diphenylhexatriene with a microviscosimeter MV-1 (Elscint, Haifa, Israel) as described previously.[6]

Table 1. Effect of Aliphatic Alcohols on Superoxide Production

Cells Incubated With*	Superoxide Release by Cells Stimulated With†		
	No Stimulant	FMLP	PMA
Buffer	0.3 ± 0.1	12.3 ± 0.6	11.1 ± 1.4
Butanol, 0.25%	0 ± 0	1.3 ± 0.5 (89%)	11.7 ± 0.2 (−5%)
Pentanol, 0.1%	0 ± 0	2.5 ± 0.6 (80%)	10.8 ± 0.4 (3%)
Isoamyl alcohol, 0.15%	0.4 ± 0	1.8 ± 0.2 (85%)	10.6 ± 0.1 (5%)

*PMNs (2.5 × 10^6) were preincubated with the indicated percentage (vol/vol) of alcohol and 10 μmol/L cytochalasin B for five minutes at 37 °C prior to the addition of stimulants.

†Cells were incubated with no stimulant, 100 nmol/L FMLP, or 1 μg/mL PMA, and superoxide release was measured for ten minutes. Superoxide release was calculated as nmol ferricytochrome c reduced/10 min/10^6 cells. Values represent the mean ± SD of duplicate determinations obtained in a representative experiment. Values in parentheses indicate percentage of inhibition. In a total of five experiments, the average percentage of inhibition by the same alcohols for FMLP was 83 ± 12, 73 ± 11, and 81 ± 6 for butanol, pentanol, and isoamyl alcohol, respectively. The alcohols produced no significant inhibition of PMA-induced superoxide release (−1 ± 1, 4 ± 4, 0.1 ± 0.3, respectively).

RESULTS

Effects of aliphatic alcohols on superoxide production and appearance of PKC in particulate fractions of PMNs. Since chemoattractants produce an increase in PKC in PMN particulate fractions,[11] we sought to determine whether this phenomenon was required for superoxide production by these agents. Cells were treated with buffer or aliphatic alcohols in the presence of cytochalasin B prior to stimulation with the oligopeptide chemoattractant FMLP or with PMA. Superoxide release was quantified in intact cells, and PKC activity was measured in subcellular fractions. Incubation of PMNs with butanol (0.25%), pentanol (0.1%) or isoamyl alcohol (0.15%) at concentrations that produced equivalent decreases in the membrane microviscosity parameter[6] (data not shown) inhibited superoxide release in response to FMLP (Table 1) by 89%, 80%, and 85%, respectively, in a representative experiment. The average inhibition of superoxide generation in five experiments with those same alcohols was 83%, 73%, and 81%, respectively. There was no significant effect on PMA-induced superoxide release. Additional experiments and previously published data[6] showed that butanol and pentanol have no effect on superoxide generation by PMA at doses of this agent ranging from 1 ng/mL to 1 μg/mL. As noted previously, FMLP did not alter PKC activity in the cytosol[11] and treatment of cells with aliphatic alcohols did not affect this (Table 2). PMA produced a profound decrease in cytosolic PKC activity, a finding that has previously been noted in PMNs and other cell types.[9,15] Butanol and pentanol enhanced the disappearance of cytosolic PKC activity produced by PMA. FMLP and PMA increased both the PKC activity found in the Triton X-100–extractable pellet and the kinase activity associated with the detergent-nonextractable particulate fraction (Table 2). Incubation of cells with the alcohols inhibited the FMLP-induced increase in PKC activity measured in both the extractable and nonextractable particulate fractions to basal or near-basal levels. In contrast, the alcohols did not significantly affect the increase in particulate fraction kinase activity produced by 1 μg/mL PMA (Table 2). The alcohols similarly did not alter the increase in this activity when submaximal doses of PMA were used (1 ng/mL to 100 ng/mL, data not shown). Table 3 shows summary data from five experiments in which the fractional stimulation of particulate fraction protein kinase activities was measured in cells incubated with buffer or the various alcohols in the presence of FMLP or PMA. Although all the alcohols diminished the increase of kinase activity induced by FMLP, in both the extractable and nonextractable fractions, they did not significantly alter PMA-induced stimulation of protein kinase activity in either particulate cell fraction.

Correlation of inhibition of FMLP-stimulated PKC redistribution with superoxide production. PMNs were incubated with buffer or with various concentrations of pentanol or butanol in the presence of cytochalasin B, and superoxide release and PKC activity in particulate fractions were measured following stimulation of the cells with FMLP or PMA. Butanol and pentanol inhibited FMLP-induced superoxide release in a dose-dependent fashion (Fig 1A). The doses of butanol and pentanol that produced 50% inhibition (ID_{50}) of superoxide were 0.16% and 0.074% respectively. In contrast, the alcohols did not alter superoxide release in response to PMA (Fig 1A). Both alcohols also produced a dose-dependent inhibition of the appearance of PKC activity in the detergent-extractable particulate fraction of PMNs in response to FMLP with ID_{50}'s of 0.11% and 0.056%, respectively, for butanol and pentanol (Fig 1B). The appearance of kinase activity in the detergent-insoluble particulate fraction of cells treated with FMLP also showed dose-dependent

Table 2. Effect of Alcohols on the Subcellular Distribution of Protein Kinase Activity in PMNs Treated With Stimulants

	Cells Stimulated With†	Protein Kinase Activity (pmol/min/10^7 Cells) Isolated From Cells Treated With‡			
		Buffer	Butanol§	Pentanol	Isoamyl Alcohol
Cell fraction*					
Cytosol	—	394 ± 3	418 ± 34	385 ± 36	424 ± 42
	FMLP	438 ± 47	418 ± 19	512 ± 3	441 ± 32
	PMA	63 ± 15	0	0	79 ± 100
Particulate fraction‖					
Extractable	—	34 ± 5	27 ± 9	22 ± 2	19 ± 5
	FMLP	63 ± 4	37 ± 1	32 ± 6	31 ± 1
	PMA	121 ± 9	117 ± 9	102 ± 6	117 ± 17
Nonextractable	—	35 ± 1	20 ± 3	23 ± 4	24 ± 5
	FMLP	52 ± 10	28 ± 8	22 ± 2	24 ± 3
	PMA	146 ± 22	137 ± 7	150 ± 31	115 ± 1

*Following incubation of cells with buffer, alcohols, and/or stimulants as indicated, the cell fractions were isolated as described in Materials and Methods.

†Where indicated, cells were incubated with 100 nmol/L FMLP for 60 seconds or with 1 μg/mL PMA for five minutes. All samples contained 10 μmol/L cytochalasin B.

‡Protein kinase activity was measured in subcellular fractions of cells that had been preincubated with cytochalasin B and buffer alone or with the indicated alcohols for five minutes at 37 °C followed by exposure to buffer, FMLP, or PMA. In nonextractable particulate fractions, protein kinase activity was measured in the absence of added calcium and lipids. Results are the mean of duplicate determinations ±SD and are expressed as pmol ^{32}P incorporated/min/10^7 cells. This experiment was performed two additional times with similar results.

§Concentrations of alcohols are as follows: butanol, .025%, pentanol, 0.1%; isoamyl alcohol, 0.15%.

‖The particulate fraction was extracted with 1% Triton X-100. The soluble material is termed extractable whereas the insoluble fraction is termed nonextractable

inhibition in the presence of increasing concentrations of the alcohols with an ID_{50} of 0.078% for butanol and 0.054% for pentanol (Fig 1C). In contrast, the PMA-stimulated appearance of PKC activity in the detergent-soluble particulate fraction (Fig 1B) and that of protein kinase activity in the nonextractable subcellular fraction (Fig 1C) were not significantly affected by any of the doses of alcohols tested.

Unlike the detergent-extractable kinase, which displayed the phospholipid and calcium requirements characteristic of PKC,[16] the detergent-insoluble protein kinase activity isolated from PMNs was not dependent upon the presence of exogenously added calcium and lipid. This may be a consequence of these components remaining in the residual membrane present in these fractions. To further characterize this detergent-insoluble kinase activity, aliquots of the detergent-nonextractable material from PMA-treated cells were incubated with EGTA to establish calcium dependence or with TFP, a phenothiazine that has been shown to inhibit both PKC and the calmodulin-dependent protein kinase.[13] EGTA (1 mmol/L) inhibited the kinase activity by 62%, whereas TFP, at concentrations of 50 and 100 μmol/L, inhibited the activity by 39% and 77%, respectively (data not shown).

DISCUSSION

In several cell types including human PMNs, phorbol ester tumor promoters translocate cytosolic PKC activity to a membrane-containing fraction.[9–11,16–19] This response may be involved in tumor promotion[16–19] as well as in the activation of the respiratory burst[9–11] by the active phorbol esters. It has been shown that the active phorbol esters directly bind to PKC in intact cells, and this enzyme is therefore thought to be the receptor for these tumor promoters.[19] Chemoattrac-

Table 3. Effect of Alcohols on Particulate Fraction–Associated Protein Kinase Activity in PMNs Stimulated With FMLP or PMA

Particulate Cell Fraction*	Cells Stimulated With†	Fractional Stimulation of Protein Kinase Activity Isolated From Cells Incubated With‡			
		Buffer	Butanol§	Pentanol	Isoamyl Alcohol
Extractable	FMLP	2.7 ± 0.1	1.7 ± 0.1	1.3 ± 0.6	1.5 ± 0.2
	PMA	5.3 ± 0.6	4.7 ± 0.4	5.1 ± 0.7	6.6 ± 0.1
Nonextractable	FMLP	2.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.5 ± 0.3
	PMA	6.9 ± 0.4	6.5 ± 0.7	8.0 ± 2.0	6.8 ± 1.3

*Following removal of cytosol, the particulate cell fractions were extracted with 1% Triton as indicated in Materials and Methods. Extractable and nonextractable portions were assayed for PKC activity (extractable) and protein kinase (nonextractable) activities.

†Cells were incubated with the indicated stimulant as described in the legend to Table 2.

‡Protein kinase activity was measured in particulate cell fractions of PMNs that had been treated with buffer or various alcohols and stimulated with FMLP or PMA as indicated in the legend to Table 2 and Materials and Methods. The fractional stimulation of protein kinase activity is equal to *S/C*, where *S* equals the amount of protein kinase present in particulate cell fractions of PMNs treated with FMLP or PMA and *C* is equal to that amount measured in the absence of a cellular stimulant (buffer alone). Results represent the average of five experiments ±SE.

§Concentrations of alcohols: butanol, 0.25%; pentanol, 0.1%; isoamyl alcohol, 0.15%.

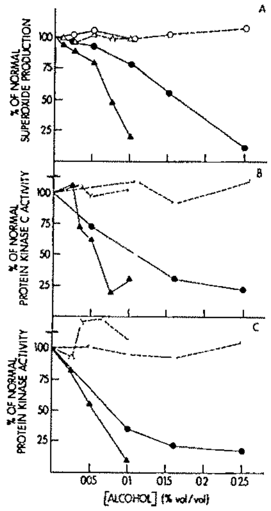

Fig 1. Dose response effects of butanol and pentanol on superoxide release (A), PKC activity in Triton X-100–extractable particulate fractions (B), and protein kinase activity in Triton X-100–insoluble particulate fractions (C) in human PMNs. Cells were preincubated with buffer and cytochalasin B alone or buffer containing butanol (circles) or pentanol (triangles) for five minutes at 37 °C followed by buffer alone or buffer containing FMLP (●, ▲) or PMA (○, △). Superoxide release was measured during a ten-minute exposure to either FMLP or PMA. Results represent the average of five experiments and are expressed as the percentage of normal FMLP- or PMA-stimulated activity in cells incubated in the absence of alcohols. The percentage of normal is calculated as *E/C* × 100, where *E* equals the activity of cells incubated in the presence of alcohols and *C* equals the activity of cells incubated in the presence of buffer alone.

tants, which stimulate superoxide production, also increase PKC activity in membrane-containing fractions of human PMNs[11]; however, chemotactic factors mediate their effects through interaction with specific cell surface receptors.[12] It can be postulated that superoxide production in leukocytes by both chemoattractants and PMA requires the activation of PKC as a component in the final common pathway leading to phosphorylation of effector proteins necessary for stimulation of the respiratory burst enzyme. To test this hypothesis, we examined the effects of aliphatic alcohols on the chemoattractant- and PMA-induced appearance of PKC activity in PMN particulate fractions. The alcohols have been shown to alter chemoattractant-mediated events at the level of the cell surface receptor, producing an increase in the affinity of the receptor, an increase in PMN chemotaxis, but a corresponding selective inhibition of chemoattractant-mediated superoxide production.[6] The present study shows that low doses of these same alcohols inhibit the FMLP-induced appearance of both PKC and the nonextractable protein kinase activity in particulate cell fractions. This effect of alcohols was dose dependent and correlated with inhibition of superoxide production. In contrast, the alcohols affected neither PMA-induced superoxide release nor enhancement of PKC activity in particulate fractions.

The protein kinase activity is the detergent-insoluble particulate fraction cannot be termed PKC since it is not stimulated by exogenously added calcium and PS. This may be due to the presence of residual lipid and calcium in the detergent-insoluble preparations. This detergent nonextractable protein kinase activity was, however, inhibited by EGTA and TFP, the latter of which is a known inhibitor of both PKC and the calmodulin-dependent, cyclic guanosine monophosphate (cGMP)-stimulated protein kinase associated with cytoskeletal fractions.[14] The insoluble kinase activity also was not stimulated further by cGMP nor by calmodulin and cGMP (data not shown), suggesting that it may be PKC in a form that does not require exogenously added Ca^{+2} or lipids. However, at this time we cannot specifically identify the kinase activity that appears in the detergent-insoluble particulate fractions of PMNs following FMLP or PMA stimulation.

The present studies suggest that stimulation of the respiratory burst in human PMNs by the chemoattractant FMLP may require the appearance of protein kinase activity in particulate fractions. We base this conclusion on the ability of the alcohols to block both the induction of the respiratory burst and the appearance of particulate-associated kinase activity mediated by chemoattractants. Indeed, NADPH oxidase, the enzyme responsible for the production of superoxide, is located in the particulate fraction of stimulated PMNs,[20] although it is not yet known if this enzyme system is directly regulated by phosphorylation. Chemotaxis is actually enhanced by doses of alcohols that inhibit the appearance of protein kinase activity in the particulate fractions[6]; so this increase in particulate fraction–associated activity is apparently not required for directed motility.

The effects of the alcohols appear to be on a chemoattractant-receptor–coupling mechanism required for activation of the respiratory burst that occurs distal to ligand binding.[6] Since the doses of alcohols used enhance the average affinity of the chemoattractant receptor as well as membrane fluidity, they could act by altering the ability of the receptor to make contact with extramembranous or transmembranous transduction units such as the guanine nucleotide regulatory unit or phospholipase C with which this receptor appears to interact.[21–25] The relationship between the affinity of the receptor and its ability to initiate the redistribution of protein kinase activity to the particulate fraction is currently unknown. However, the data suggest that the affinity state of the chemoattractant receptor reflects its ability to mediate such a redistribution of kinase activity and that this process may play a role in determining whether PMNs migrate or release cytotoxic products in response to chemoattractants.

The alcohols did not have nonspecific toxic effects since neither superoxide production nor changes in particulate fraction–associated PKC alterations induced by PMA were affected. One would not expect to see an effect of the alcohols on these PMA-induced functions since phorbol esters directly bind to and activate PKC,[19] thus bypassing the more complexly regulated receptor-coupling mechanisms operating in chemoattractant-mediated stimulation.[25–27] The apparent requirement for the appearance of kinase activity in the particulate fraction for stimulation of superoxide release suggests that subcellular redistribution of protein kinases during cellular activation leads to phosphorylation of membrane-associated proteins. This process may be a necessary step in the biochemical transduction of chemoattractant-receptor–mediated signals for activation of the respiratory burst.

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T Cell Receptor Gene Rearrangements Define a Monoclonal T Cell Proliferation in Patients With T Cell Lymphocytosis and Cytopenia

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We have used probes from the T cell receptor β and γ chain loci to investigate the clonality of T lymphocytes in eight patients with T cell lymphocytosis and cytopenia (TCLC). This syndrome, which is strongly associated with rheumatoid arthritis, is characterized by peripheral blood and bone marrow lymphocytosis and neutropenia, red cell aplasia, or both. By means of T cell monoclonal antibodies and flow cytometry, T lymphocytes from patients with this syndrome have been shown to have characteristic immunologic features. Investigators have disagreed as to whether the syndrome represents a T cell malignancy or a more benign immunologic disorder. DNA from five of five patients with symptomatic "classic" T cell lymphocytosis with cytopenia demonstrated unique rearrangements of the T cell receptor β chain locus, whereas neither of two patients with atypical features showed rearrangement. In addition, we found evidence for γ chain rearrangement in those DNAs with clonal β chain rearrangement. We thus postulate that the classic form of this syndrome is associated with a monoclonal proliferation of T cells. Its potential relationship to T cell chronic lymphocytic leukemia is discussed.

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THE SYNDROME of T cell lymphocytosis associated with granulocyte or erythrocyte aplasia is a rare but distinct clinical entity.[1-4] Patients initially may have recurrent fever and infections associated with neutropenia or symptoms of fatigue related to anemia. Diagnosis is sometimes made by routine blood testing in asymptomatic patients There is a strong association of this syndrome with rheumatoid arthritis (RA); in the largest reported series of these patients, one third had RA.[2] Physical findings include splenomegaly, but hepatomegaly or lymph node enlargement is rare. Most patients have neutropenia, a few patients have erythrocyte aplasia, and rare patients have both. Thrombocytopenia is unusual. All patients have a peripheral blood lymphocytosis with an associated infiltration of their bone marrow with mature-appearing lymphocytes. In neutropenia, the marrow usually shows a predominance of early myeloid forms; in red cell aplasia, there may be a complete absence of erythrocyte precursors in the marrow. The course of the disease in most patients is chronic and only the minority require treatment, although some patients have died from their disease.

Previous studies have used T cell functional studies as well as monoclonal antibodies and flow cytometry to characterize the peripheral blood lymphocytes in these patients.[5-8] The T cells display a characteristic phenotype. They are CD8 (Leu2,OKT8) positive, characteristic of suppressor T cells, but they are negative, or only weakly positive, for a panel of pan-T cell CD5 markers. They also usually express the Leu7 and H366 antigens and receptors for the Fc portion of IgG, markers typically associated with natural killer cell activity.

The uniformity of the expression of these characteristic T cell antigens in the setting of the lymphocytic infiltration of the bone marrow and peripheral blood has led many investigators to postulate that this represents a monoclonal disorder related to chronic lymphocytic leukemia.[9] However, the indolent clinical course of the disease in most patients and its strong association with rheumatoid arthritis have suggested to others a more benign immunologic disorder.[2] Many of these patients have been given a diagnosis of Felty's syndrome. However, these patients can be distinguished from patients with "classic" Felty's syndrome by surface marker studies of peripheral T cells; patients undergoing splenectomy can be distinguished from those with Felty's syndrome by histologic examination of their spleens.[4]

The availability of probes for T cell receptor genes offers an opportunity to help resolve this controversy. The genes for the β chain of the T cell receptor have been cloned and have been shown to rearrange in a manner analogous to the immunoglobulin genes in B lymphocytes.[10] The immunoglobulin genes are encoded on discontinuous genes. During the course of B cell differentiation, these genes undergo rearrangement to form functional continuous genes which encode for antibody. These rearrangements are detectable by DNA blotting techniques, and rearrangement studies have been used to establish monoclonality of B cell tumors,[11] to diagnose malignancy in the face of confusing histology,[12] and to show that some classic lymphomas are in fact biclonal.[13] We have used the rearrangement of the β chain genes of the T cell receptor as a marker for monoclonality of T cells in an analogous manner. We have performed Southern blot analysis of the T cell receptor β chain genes in six patients with classic T cell lymphocytosis and cytopenia (TCLC) and in two patients with an atypical presentation. We have demonstrated unique rearrangements of the β chain genes in five of five patients symptomatic with the classic syndrome. We did not find rearrangements in a sixth patient from whom only remission blood was available or in two patients with atypical features.

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Table 1. Clinical Characteristics of Patients with TCLC

Case	Sex	Age	Initial Complaint	H/O RA	Affected Cell Line	Treatment	Survival (yr)
1	F	46	Routine	+	Eryth Neut	Spl Aza, Chl MCAB	>10
2	F	68	FUO	+	Neut	Chl, Pred	>1
3	M	64	Fatigue	Ser*	Eryth	Pred	3 (LFU)
4	F	48	UTI Fatigue	+	Neut	Chl	>3
5	M	55	Abdominal Pain	−	Neut	Chl	>6
6	F	59	Infection Phlebitis	+	Neut	Spl	>6
7	M	87	Enteritis	−	Pan	None	<1
8	M	53	UTI Pneumonia	−	Neut	None	>3

RA, rheumatoid arthritis; FUO, fever of unknown origin; UTI, urinary tract infection; Eryth, erythrocytes; Neut, neutrophils; Spl, splenectomy; Aza, azathioprine; Chl, chlorambucil; Pred, Prednisone; And, androgens; MCAB, monoclonal antibody; LFU, lost to follow-up.

*Serology only.

The γ chain genes from the mouse and from humans have also been cloned recently.[14,15] In the mouse, functional somatic rearrangement has been shown to occur primarily in cytotoxic T cells.[16] Our analysis of the DNAs from TCLC patients with β chain rearrangement showed evidence of γ chain rearrangement.

MATERIALS AND METHODS

DNA extractions were performed as described previously.[16] In brief, high mol wt DNA was prepared from spleen cells from patient 1 and from E rosette-purified peripheral blood T cells obtained from the remaining patients. When available, cells used were from patients both when they were symptomatic and when they were in remission. DNA samples from B cell lines from the same patients were used for controls. Cells were incubated overnight at 37 °C in 0.5% sodium dodecyl sulfate (SDS) and 0.2 mg/mL Proteinase K. This was followed by phenol-chloroform extraction and ethanol precipitation. DNA was digested with appropriate restriction enzymes, size-fractionated by agarose gel electrophoresis, and transferred onto nitrocellulose paper by the method of Southern.[17] Filters were hybridized to nick-translated, ^{32}P-labeled probes of the T cell receptor β and γ chain loci and washed at 55 °C in 0.1% SDS/0.15 mol/L of NaCl and 0.0015 mol/L of Na citrate prior to autoradiography.

RESULTS

Clinical profile and lymphocyte phenotype. The clinical characteristics and peripheral blood lymphocyte phenotyping of the eight patients studied have been described previously,[2] and are outlined in Tables 1 and 2. Patients 1 through 6 were considered to have had classic T cell lymphocytosis in that they had peripheral blood lymphocytosis, bone marrow lymphocytosis, cytopenia, and the typical immunologic phenotype. Blood was available in case 4 only when the patient was in clinical remission. Cases 7 and 8 were atypical in that the patients had no peripheral blood lymphocytosis but had marrow infiltration and the same T cell phenotype in their bone marrow.

Detection of T cell receptor β chain rearrangement. The organization of the β chain locus is diagrammed in Fig 1. The two constant regions (Cβ1 and Cβ2) are physically linked and lie approximately 8 kilobases (kb) from one another.[18] The probe used to analyze genomic DNA was a 1.0 kb *Bgl* II to *Bgl* II fragment from Cβ2. The two constant region genes are highly homologous in the coding region; therefore, this fragment hybridizes equally to both genes as demonstrated in Fig 1. On digestion of DNA with *Eco* RI and hybridization to the probe, two restriction fragments are predicted: a 10.8-kb fragment resulting from hybridization to Cβ1, and a 3.7-kb fragment resulting from hybridization to Cβ2. Because there is an *Eco* RI site 3′ to the Jβ2 genes, this probe will only detect rearrangements involving the joining (J) region of Cβ1. Rearrangements involving the Cβ2 genes can be detected on hybridization of the same probe to *Hind* III digests of genomic DNA. This digest results in three germline bands of 7.5, 6.5, and 3.4 kb. The first band represents hybridization to a fragment which includes the J region of Cβ2, and allows detection of rearrangements into that locus.

We detected unique rearrangements of the T cell receptor β chain in DNA from all five symptomatic patients with

Table 2. Initial Laboratory Findings in TCLC Patients

	Peripheral Counts					Lymphocyte Phenotype				
Case	Hb (g/dL)	Neut ($\times 10^9$/L)	Lymp ($\times 10^9$/L)	PLt ($\times 10^9$/L)	BML (%)	E ($\times 10^9$/L)	γFc^R	CD5	CD4	CD8
1	8.5	1.0	9.0	315	30	85	65*	24*	10*	81*
2	10.4	0.5	11.8	235	60	69	ND	28*	4*	85*
3	4.4	2.5	7.9	445	45	88	68*	27*	13*	77*
4	10.9	0.5	4.0	130	30	78	65	40	ND	70
5	10.8	0.6	11.5	257	30	83	97*	16*	4*	95*
6	13.3	0.3	5.5	100	36	87	92*	34*	9*	82*
7	8.0	0.8	1.9	62†	30	89	ND	56	28	64
8	12.7	0.5	1.9	140	37	50	ND	45	34	23
NL	12–17	1.8–7.0	1.5–4.0	150–440	<15	65	—	84%*	44	25
						± 12		± 20	± 11	± 13

Neut, neutrophils; Lymp, lymphocytes; Plt, platelets; BML (%), percentage of bone marrow lymphocytes; E^+, E rosette positive; γFcR, Fc receptor positive; CD5, OKT1, Leu1; CD4, OKT4, Leu3; CD8, OKT8, Leu 2; NL, normal range, ND, not done.

*Value obtained on E rosette-sorted cells.

†Antiplatelet antibodies were present.

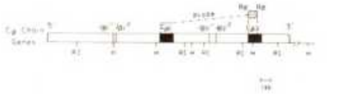

Fig 1. Organization of the human T cell receptor β chain genes. The two constant region genes (Cβ1 and Cβ2) are shown. Southern blot analysis was performed using a *Bgl* II–*Bgl* II probe from Cβ2 which hybridizes equally to both constant regions. DNA was digested with *Eco* RI or *Hind* III. The restriction sites for those enzymes are shown.

classic TCLC. Three of these cases are illustrated in Fig 2. In each case, one or more rearranged bands are seen in the DNA from patients at the time of their symptomatic illness; these bands are absent in DNAs from patients in remission (Fig 2; T cells, CR). Case 4, from whom only remission blood was available, revealed no rearrangement. The other five cases all showed rearrangement of Cβ2; only two revealed rearrangement of Cβ1. As discussed below, interpretation of Cβ1 rearrangement was hampered by the presence of a frequent unexplained band in digests of normal DNA.

The analysis of case 1 is unusual in that four rearranged bands are apparent. This is consistent with either the presence of at least two independent clones of T cells or with multiple rearrangements occurring in the same cell. The other cases were definitely consistent with a single clone of abnormal cells because the DNAs revealed only one or two rearranged bands.

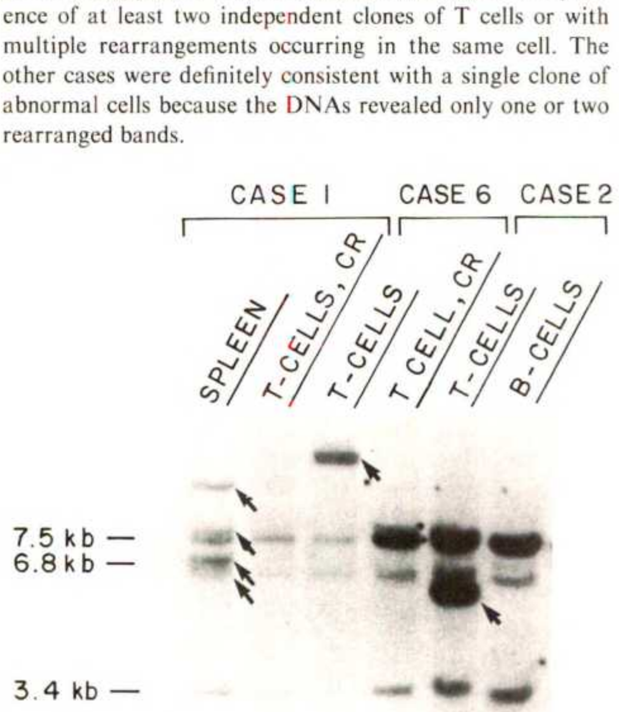

Fig 2. Evidence for β chain gene rearrangement from Southern blot analysis of DNAs from patients with TCLC. *Hind* III-digested DNAs were size-fractionated on 0.8% agarose gels and blotted on nitrocellulose as described in the Materials and Methods section. Filters were hybridized to the Cβ2 probe. Autoradiogram of three cases reveals bands indicative of somatic rearrangement of Cβ2 in all three cases. Rearranged DNA bands are indicated by arrows. Case 1 shows four discrete rearrangements, indicating a proliferation of at least two different clones. Cases 2 and 6 show evidence of a single clone, in that each sample shows only one rearranged band. In two cases, remission samples (CR) are shown and demonstrate disappearance of the monoclonal rearrangements. No remission sample was available on patient 2; a B cell line from the same patient is shown as a control.

Case 6 demonstrates the difficulty of interpreting rearrangements of Cβ1. Although this patient had a clear rearrangement of Cβ2, which in remission disappeared in the expected fashion, we noted the appearance of what appeared to be a single rearrangement of Cβ1 arising de novo in this patient in remission (Fig 3). On further analysis, we noted the frequent appearance of a similar 9-kb band, corresponding to apparent rearrangement of Cβ1, in digests of DNA from patients with unrelated disorders and in DNA from peripheral blood cells of 11 of 11 normal individuals. Minden et al observed an aberrant band in *Eco* RI digests of genomic DNAs from fibroblasts,[19] which may represent a similar finding. The DNA sequences represented by this band remain to be determined.

Detection of T cell receptor γ chain rearrangement. There are two human γ chain constant region genes.[15] We have shown that mature T cells frequently show deletion of one of the constant region genes that probably occurs in the course of somatic rearrangement of the γ chain genes.[15] DNAs from the TCLC patients were analyzed with a probe from the Cγ1 constant region to see if rearrangement of the γ chain genes, with subsequent deletion of the other gene, could be inferred from the loss of one hybridizing band on Southern blot analysis. Partial loss of one band was seen on hybridization of the γ chain probe to DNAs from patients in their symptomatic phase of the illness (Fig 4, T cells); such a deletion is not observed on analysis of DNAs from patients in remission or of DNAs from B cell lines derived from the same patients. One exception is case 1, in which there is still deletion of one of the constant region genes in DNA in remission, but no deletion in B cells. The possible significance of this will be discussed below.

DISCUSSION

The finding of discrete rearrangements of the immunoglobulin genes in B cell proliferative syndromes has been widely viewed as a marker of monoclonality and, by inference, of malignancy.[11,12] We have applied the same techniques to the examination of a T cell lymphoproliferative disorder and have shown monoclonal rearrangements of the T cell receptor genes. By analogy, we postulate that, in its classic form, TCLC usually represents a clonal expansion of a single or a limited number of T lymphocytes.

Whether this disease should consequently be labeled a "malignancy" is questionable, however. "Benign monoclonal gammopathy" is a syndrome in which a proliferation of a single clone of B cells gives rise to an abnormal circulating immunoglobulin. A small percentage of patients with this syndrome develop multiple myeloma; in the majority, however, it is clinically silent. TCLC may represent a similar disorder of T cells. However, the findings in this syndrome mimic those in B cell chronic lymphocytic leukemia (CLL) in many ways: TCLC is associated with a peripheral blood lymphocytosis, marrow infiltration with lymphocytes, and cytopenia. The finding of monoclonality in this disorder makes it highly analogous to B cell CLL and suggests that TCLC may in fact be a form of T cell CLL.

Two features make TCLC clinically distinct from CLL, however. One is the strong association with RA, a known

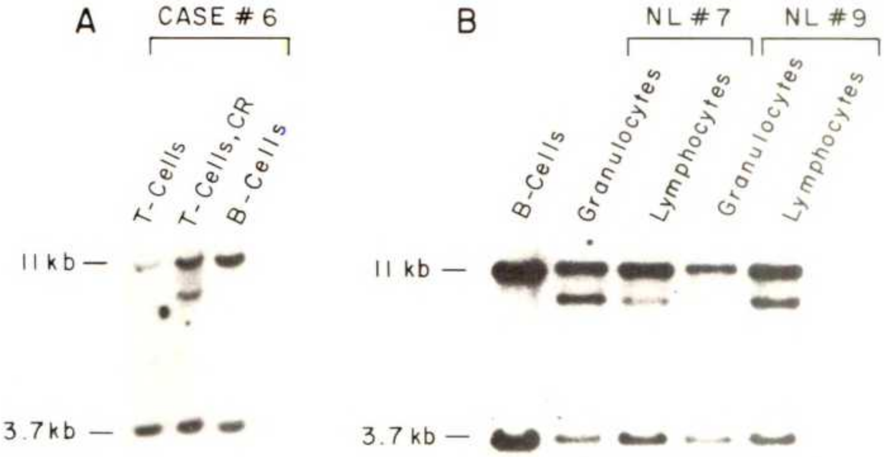

Fig 3. Presence of an aberrant band on Southern analysis of *Eco* RI digests of normal DNA. *Eco* RI-digested DNA was analyzed as explained in Fig 1. DNA from patient 6 when in remission (CR) shows a single unique nongermline band which was not present in the symptomatic (S) phase of the illness. The same band is seen on similar analysis of both lymphocytes and granulocytes from two normal individuals. It is absent in DNA from a B cell line.

autoimmune disease. The other is the observation that the syndrome is not associated with the clinical progression expected with lymphoproliferative diseases in general, and in T cell syndromes in particular. In addition, in some cases, the bone marrow is involved with similar cells, but we have been unable to detect a clonal population of peripheral blood T cells. It may be that in those cases without peripheral lymphocytosis, the clonal population is too small to detect in the peripheral blood. Alternatively, these patients may have a monoclonal population that does not have β chain rearrangement. We have not looked for α chain rearrangement in the DNAs of these patients. Finally, although these cases may simply represent a different entity, they may represent an early stage of the classic disorder from which a clonal disease may emerge. Longitudinal studies and comparisons to similar studies on patients with Felty's syndrome and patients with other autoimmune diseases may help to clarify this further.

The finding of an aberrant band on analysis of Cβ1 in normal cells is unexplained. The band may represent a true somatic rearrangement, an unexplained cross-hybridizing sequence, or an artifact. Although the significance of this finding in biological terms is not clear, in technical terms, it is extremely important to the correct interpretation of similar studies. This "normal" band may represent a large fraction of total peripheral blood mononuclear cells (in one normal individual, it appeared in equimolar concentration to the germline bands), and may be very misleading in the evaluation of gene rearrangement studies. Normal T cells from the patient, either from remission samples or from phenotypically normal cells sorted from early samples, must be used as controls for Cβ1 rearrangements. We have not noted similar stereotypic rearrangements of Cβ2, and interpretation of rearrangements involving this locus is not difficult.

The suggestion that DNA rearrangement has also occurred in the γ chain locus offers an intriguing avenue for further investigation. Although γ chain rearrangement has been shown to occur at the DNA level in all classes of T cells,[15,20] functional rearrangement leading to mRNA transcription has been shown to occur almost exclusively in murine cytolytic T cells.[20] This may help to clarify the phenotype of these cells. In vitro studies have demonstrated antibody-dependent cytotoxicity and natural killer activity[5,21] and apparent suppression of marrow growth by subsets of T cells in these patients.[6,22] This may suggest that these cells are functional cytotoxic cells that mediate the cellular aplasia characterizing this syndrome, but soluble factor inhibition cannot be excluded. Evidence of functional γ chain production by these cells would suggest that these cells are indeed cytolytic T cells and support the former contention.

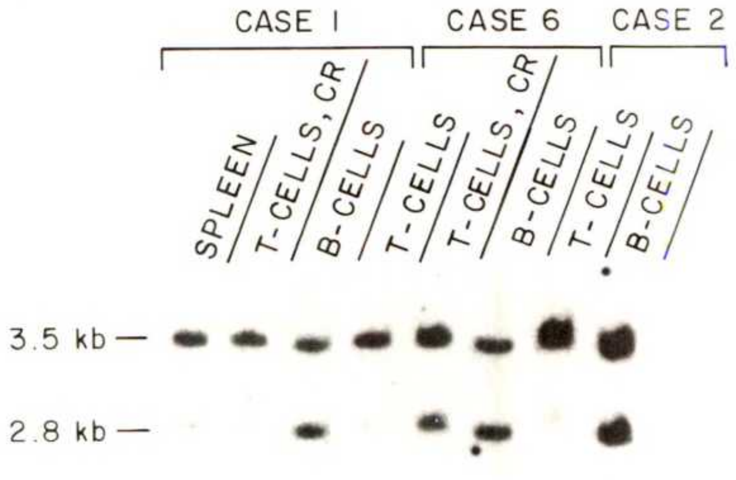

Fig 4. Evidence for γ chain rearrangement from Southern blot analysis of DNA from patients with TCLC. DNAs were digested with *Hind* III, and filters were hybridized to a probe from the Cγ1 locus, yielding two hybridizing bands of 3.5 kb and 2.8 kb in germline DNA. Samples of DNA from each patient during the symptomatic phase of illness show almost total disappearance of the 2.8-kb band, indicating deletion of one of the constant region genes. This is also seen on a remission sample from patient 1, but not on a remission sample from patient 6. DNA from B cell lines from all three patients are shown; in each case, the 2.8-kb hybridizing band is of an intensity equal to that of the 3.5-kb band, indicating a germline configuration without deletion.

Further analysis of the functionality and specificity of the unique T cell receptor gene rearrangements should help to elucidate this further.

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Partial Spectrin Deficiency in Hereditary Pyropoikilocytosis

By Theresa L. Coetzer and Jiri Palek

Hereditary pyropoikilocytosis (HPP) is a severe hemolytic anemia in which an instability of the red cell membrane skeleton has been correlated with structural and functional defects of spectrin. We now report that 13 unrelated HPP subjects have ~30% less spectrin than normal as evidenced by a decreased spectrin/band 3 ratio. We also examine the role of spectrin degradation as an underlying cause of this partial spectrin deficiency. Our studies demonstrate that the reduced spectrin content of HPP red cells remains constant during in vivo aging of the cells in the peripheral blood, as well as during in vitro incubation. Furthermore, immunoblotting experiments using an affinity-purified antispectrin antibody indicate that there is no loss of spectrin during membrane preparation and also that neither whole HPP red cells nor ghosts nor cytosol contains any abnormal spectrin degradation products. These data suggest that spectrin is not degraded and that it is stable on the membrane of the circulating HPP red cell. In contrast, however, incubation of free spectrin with a lysate of nucleated erythroid precursor cells indicates that HPP α I/46 spectrin, but not HPP $\alpha^{I/74}$ spectrin, is more susceptible to proteolytic degradation than a control. These data imply that the decreased spectrin content of HPP is not due to a single defect but that a more complex mechanism is involved. In HPP Sp $\alpha^{I/46}$ subjects, an increased proteolytic degradation in bone marrow erythroid precursors of cytosolic spectrin, prior to its assembly on the membrane, could contribute toward the partial spectrin deficiency.

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HEREDITARY pyropoikilocytosis (HPP) is a relatively rare, severe congenital hemolytic anemia characterized by striking micropoikilocytosis and microspherocytosis, autosomal recessive inheritance, and a thermal instability of the red cells.[1] The red cell membrane skeleton, which consists primarily of spectrin, actin, and band 4.1, is markedly unstable in HPP subjects when subjected to shear stress.[2] This skeletal instability may be correlated with defective spectrin dimer–dimer self-association, resulting in an increased amount of spectrin dimers as opposed to tetramers or oligomers in the skeleton.[2] On a structural level, the 80,000 dalton α I domain of spectrin, which represents the dimer–dimer self-association site, is altered in HPP subjects, as evidenced by limited tryptic digestion.[3-5] On this structural basis, two subtypes of HPP may be distinguished depending on the size of the major abnormal α I peptide generated. In HPP, the normal α I 80,000-dalton domain is decreased or absent, and there is a concomitant increase in either a 74,000-dalton peptide, representing HPP Sp $\alpha^{I/74}$, or a 46,000-dalton fragment, representing HPP Sp $\alpha^{I/46}$.[3,4,6] Another feature of the HPP spectrin molecule is an increased susceptibility to thermal denaturation as measured by circular dichroism,[7] extractability,[8] and aggregation.[9]

A decrease in the amount of spectrin present in the red cell membranes of three HPP subjects has also been noted.[9,10] In this article, we confirm and extend this observation by documenting a partial spectrin deficiency in a large number of HPP patients (n = 13) and by comparing the membrane spectrin content of HPP to HE Sp $\alpha^{I/74}$ subjects and to asymptomatic carriers of HPP. In addition, we examine the role of spectrin degradation as an underlying cause of this partial spectrin deficiency in HPP. Our results indicate that the decreased spectrin content in HPP is not due to excessive degradation of the abnormal spectrin in peripheral blood, but that it may be partly due to increased proteolysis of spectrin prior to its assembly on the membrane in bone marrow erythroid precursors.

MATERIALS AND METHODS

Subjects. Thirteen HPP patients from 12 unrelated families were studied. The clinical diagnosis was based on: (a) the characteristic red cell morphology of striking poikilocytosis and microspherocytosis associated with an MCV ranging from 44 to 80 fL; (b) an increased susceptibility of the cells to thermal fragmentation and budding which occurred at 45 °C to 46 °C as opposed to 49 °C for controls; (c) an autosomal recessive mode of inheritance; and (d) an absence of identifiable abnormalities in glycolytic enzymes and hemoglobin (with the exception of patient C. E. who was homozygous for Hb C) known to be associated with nonspherocytic hemolytic anemia.[1,11] Two of the subjects (S. W. and B. S.) were white; the rest were black.

On a biochemical basis, all of the HPP subjects exhibited a marked instability of the red cell membrane skeleton when exposed to mechanical or shear stress, as well as a defective spectrin dimer–dimer self-association as evidenced by an increased amount of spectrin dimers in the membrane.[2] The proportion of spectrin dimers in the crude 4 °C spectrin extract ranged from 49% to 76% of the total spectrin dimer–tetramer pool as compared with control values of 6.0% ± 3.7% (n = 22). Limited tryptic digestion of spectrin from the HPP subjects indicated an abnormality in the α I domain of spectrin, which contains the spectrin dimer–dimer self-association site. The peptide patterns generated showed a decreased amount of the normal α I 80,000-dalton peptide and a concomitant increase in either a 74,000-dalton or 46,000-dalton peptide.[3,4] This allowed classification of the patients into subgroups and, by using the recently proposed nomenclature,[6,12] they are referred to as HPP Sp $\alpha^{I/74}$ or HPP Sp $\alpha^{I/46}$. One patient is heterozygous for both defects and is referred to as HPP Sp α I/74, 46.

The clinical and biochemical data on the hereditary elliptocytosis (HE) patients have been reported previously.[13-15] Asymptomatic carriers of HPP were identified either by the presence of an abnormal α I 74,000- or 46,000-dalton peptide or by an increased spectrin dimer content in the membrane, or both.

Venous blood was collected from the subjects, their relatives, and normal control donors in tubes containing acid citrate dextrose as

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anticoagulant. The samples were cooled and transported on ice in insulated containers to Boston, where they were kept at 4 °C and analyzed either immediately or within three days.

Erythrocyte membrane preparation. Erythrocyte ghosts were prepared by hypotonic lysis at 4 °C of washed red blood cells in 3 mmol/L of $NaPO_4$, pH 8, 0.1 mmol/L of EDTA, and 0.1 mmol/L of phenylmethylsulfonylfluoride (PMSF), a protease inhibitor included to minimize proteolysis.[16] Whole cells were prepared for electrophoresis by solubilization of washed erythrocytes in 10 mmol/L of Tris HCl, pH 8, 1 mmol/L of EDTA, 5% sodium dodecyl sulfate (SDS), 5% β mercaptoethanol, and 5% sucrose, and by boiling for three minutes. Erythrocyte cytosol was obtained by lysis of washed red cells in 5 vol of lysis buffer and centrifugation at 150,000 g for 30 minutes at 4 °C. The supernatant was solubilized as described for whole cells.

Protein electrophoresis and quantitation of spectrin content. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5.6% polyacrylamide gels and stained with Coomassie blue.[17] Protein concentrations were measured by the Bio Rad protein assay using bovine serum albumin (BSA) as standard. The amount of spectrin on the membrane was expressed as a ratio of spectrin to band 3 and was quantitated by densitometry of the stained gels at 540 nm and integration of the surface area under the spectrin and band 3 peaks. An alternative method of eluting Coomassie blue stain from the gel bands by 25% pyridine (vol/vol) was also used in some cases.[18]

Preparation of spectrin. Spectrin was extracted from erythrocyte ghosts by low ionic strength incubation in 0.1 mmol/L of $NaPO_4$, 0.1 mmol/L of EDTA, 0.1 mmol/L of dithiothreitol (DTT), pH 8 for 16 hours at 4 °C or for 30 minutes at 37 °C. The samples were centrifuged at 150,000 g for 30 minutes at 4 °C, and the supernatants were collected. Spectrin dimers were purified from crude control 37 °C extracts or from crude HPP 4 °C extracts by density gradient centrifugation for 16 hours at 155,000 g and 4 °C on a linear 5% to 20% sucrose gradient in either 10 mmol/L of $NaPO_4$, 20 mmol/L of NaCl, 0.1 mmol/L of EDTA, 0.1 mmol/L of DTT, and 0.1 mmol/L of NaN_3, pH 7.4 for antibody production, or in 5 mmol/L of Tris-HCl, 120 mmol/L of KCl, and 30 mmol/L of NaCl, pH 7.2, for spectrin incubation experiments.

Preparation of affinity-purified antispectrin antibody. A polyclonal antispectrin antibody was raised in New Zealand white rabbits by multiple subcutaneous injections of pure control spectrin dimers (200 to 300 μg of spectrin emulsified 1:1 with complete Freund's adjuvant) followed by two similar injections at two-week intervals with antigen in incomplete Freund's adjuvant. Serum was collected, and the antibody was affinity-purified by applying the antiserum to an Affigel 15 column (Bio Rad) containing 1 mg of spectrin per milliliter of gel. The column was washed with PBS (5 mmol/L of $NaPO_4$, 150 mmol/L of NaCl, 1 mmol/L of NaN_3, pH 7.5), and the antibody was eluted with 0.2 mol/L of glycine, pH 2.3. The fractions were immediately neutralized with $NaPO_4$, and those with the highest absorbance at 280 nm were pooled, dialyzed against phosphate-buffered saline (PBS), and stored at −20 °C.

Preparation of anti-α I spectrin antibody. The 80,000-dalton α I domain of spectrin was isolated by the procedure of Hanspal and Ralston,[19] with minor modifications. A crude low ionic strength spectrin extract was digested with TPCK trypsin (Worthington, Malvern, Pa) at 1:100 wt/wt for 20 hours at 0 °C. The digest was applied to a 1.9 × 70-cm Sepharose 4B column, and the peak containing the 80,000-dalton fragment was again concentrated and applied to a 3 × 75-mm high-performance liquid chromatography (HPLC) column of Bio Sil diethylaminoethanol (DEAE) equilibrated with 10 mmol/L of $NaPO_4$, pH 7.2. The column was developed with a gradient of 0 to 0.6 mol/L of NaCl. The 80,000-dalton α I domain obtained by this procedure was homogeneous on SDS-PAGE. A polyclonal anti-α I antibody was raised in rabbits as described above for the production of an antispectrin antibody.

Immunoblot analysis. Samples were subjected to SDS-PAGE on 5.6% or 4.5% to 15% gradient polyacrylamide SDS gels.[17] The proteins were electrophoretically transferred to nitrocellulose paper in 25 mmol/L of Tris, 192 mmol/L of glycine, 20% methanol, pH 8.3 for 20 hours at 50 V and 4 °C in a Bio Rad transblot apparatus.[20] The nitrocellulose was incubated in 20 mmol/L of Tris HCl, 0.9% NaCl, pH 7.5 (Tris-buffered saline, TBS) containing 3% BSA for 60 minutes at 22 °C. Antibody (affinity-purified antispectrin or anti-α I spectrin) was added at a dilution of 1:200 to 1:1000, and the blot was incubated for 60 minutes at 22 °C. The nitrocellulose was washed five times over a 30-minute period with TBS containing 0.05% Tween-20. A second antibody (peroxidase-conjugated goat anti-rabbit IgG, Cappel, Cochranville, Pa) was added at a dilution of 1:1000 in TBS containing 3% BSA and incubated for 60 minutes at 22 °C. The blot was washed as before and incubated with TBS containing 0.0075% H_2O_2 and 0.05% 4 chloro-1-naphthol (dissolved in methanol at 3 mg/mL, SIGMA). Development of a purple color allowed visualization of spectrin or spectrin fragments. The sensitivity of the immunoblotting technique is in the 10-ng range, which would allow detection of 0.5% of the membrane spectrin content under the experimental conditions used.

Incubation of erythrocytes. Washed erythrocytes from control and HPP subjects were suspended in 9 vol of 50 mmol/L of glycylglycine, 100 mmol/L of NaCl, 5 mmol/L of KCl, 2 mmol/L of $MgCl_2$, 2 mmol/L of $CaCl_2$, 0.5 mmol/L of adenine, 12 mmol/L of inosine, and 100 mmol/L of glucose, pH 7.4,[9,21] and incubated at 37 °C for 16 hours in a shaking waterbath. These conditions allowed the cells to maintain adequate ATP and GSH levels. After incubation, membranes were isolated, and the spectrin content was analyzed by SDS-PAGE and immunoblotting techniques as described.

Fractionation of red cells by density gradient centrifugation. Blood samples were obtained from control and HPP subjects with a high reticulocyte count (14% to 26% reticulocytes). The red cells were washed and subjected to density gradient centrifugation on 75% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). The gradients were fractionated, and each fraction was monitored for red cell morphology, percentage of reticulocytes (quantitated on brilliant cresyl blue-stained smears), glucose-6-phosphate dehydrogenase (G6PD) activity (analyzed by a Sigma kit), and spectrin content as described above.

Isolation of nucleated erythroid precursor cells. Nucleated erythroid precursor cells were isolated from the spleens of anemic rats by the method of Hanspal and Palek (M. Hanspal and J. Palek, unpublished observations and personal communication, January 1985). Anemia was induced in Sprague Dawley rats by intraperitoneal injections of 1% aqueous phenylhydrazine.[22] The rats were killed, and their spleens were placed on ice in Hanks balanced salt solution (HBSS) with no calcium or magnesium (Whittaker, M.A. Bioproducts). A cell suspension was obtained by slicing the spleens with a scalpel, disrupting the tissue with flat-tipped forceps, and then passing the fragments through a 50-mL syringe. After filtration through polyethylene gauze (Spectramesh) with a mesh size of 202 μm (Spectrum Medical Industries, Inc, Los Angeles, Calif), the cells were collected by centrifugation at 1,500 g for five minutes at 4 °C. The cells were washed twice with HBSS, resuspended and layered onto an equal volume of Ficoll-Paque (Pharmacia), and centrifuged at 800 g for 20 minutes at 4 °C to remove lymphocytes. The pellet was washed twice with HBSS and resuspended, and 1 mL of cell suspension was then layered onto 30 mL of a discontinuous Percoll gradient consisting of layers of 45%, 55%, 65%, 77%, and 90% Percoll in HBSS. The gradient was centrifuged at 5,000 g for 20 minutes at 4 °C, and cells sedimenting in the 65% and 77% Percoll layers were collected, washed twice with HBSS, and stained with benzidine/hematoxalin to identify nucleated erythroid precursor cells. White cell contamination was 2% to 4%.

Incubation of spectrin with nucleated red cell proteases. Crude 4 °C spectrin extracts as well as purified spectrin dimers were prepared from control and HPP subjects as described. The spectrin samples were incubated with freeze thawed nucleated erythroid precursors in 5 mmol/L of Tris HCl, 120 mmol/L of KCl, 30 mmol/L of NaCl, 1 mmol/L of $MgCl_2$, 0.1 mmol/L of $CaCl_2$, 1 mmol/L of DTT, 1 mmol/L of ATP, pH 7.2 at 37 °C for up to four hours. Aliquots were removed at 30, 60, 150, and 240 minutes and centrifuged at 30,000 g for 15 minutes; the supernatants were subjected to SDS-PAGE and immunoblotting techniques as described.

RESULTS

Spectrin content is diminished in HPP erythrocyte membranes. The membrane spectrin content is expressed as a spectrin/band 3 ratio and was measured by scanning densitometry of Coomassie blue-stained SDS polyacrylamide gels and confirmed by pyridine elution of the gel bands in some cases. Figure 1 shows the spectrin/band 3 ratio in normal control individuals, HPP subjects, asymptomatic carriers of HPP, and HE subjects who have the same structural (Sp $\alpha^{I/74}$) and functional (spectrin dimer–dimer self-association) defect. In control membranes, spectrin and band 3 were present in approximately equal amounts, as indicated by a mean spectrin/band 3 ratio of 1.00 ± 0.09 (mean ± SD). HE patients, as well as the carriers, had a normal spectrin content, with ratios of 0.97 ± 0.05 and 0.98 ± 0.07, respectively. In contrast, all 13 HPP patients studied have a decreased spectrin/band 3 ratio with a mean value of 0.74 ± 0.05. There was no significant difference between the various subtypes of HPP as evidenced by ratios of 0.75 ± 0.05 for HPP Sp $\alpha^{I/74}$ (n = 7), 0.72 ± 0.05 for HPP Sp $\alpha^{I/46}$ (n = 5), and 0.71 for the double heterozygote HPP Sp $\alpha^{I/74,46}$.

To rule out the possibility of spectrin loss during membrane preparation, samples of ghosts were taken at each stage of preparation and examined for spectrin content by quantitation of the spectrin/band 3 ratio. Data indicated that there was no loss of spectrin during HPP membrane preparation (not shown). Furthermore, no spectrin was present in the cytosol after lysis of the HPP cells, as evidenced by immunoblotting experiments (Fig 2).

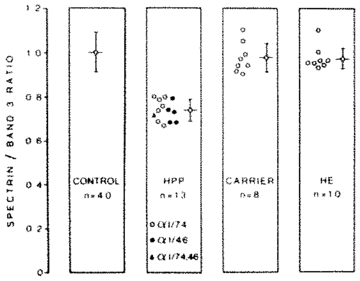

Fig 1. Spectrin content of red cell membranes. Spectrin was quantitated in membranes from control individuals, HPP subjects, asymptomatic carriers, and HE subjects with a spectrin dimer–dimer association defect by densitometry of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels or by elution with pyridine. The spectrin content is expressed as a spectrin/band 3 ratio; for each group, the mean value ± SD is indicated.

HPP spectrin is not degraded in circulating red blood cells. To investigate whether the decreased spectrin content of HPP red cell membranes is related to a loss of spectrin during in vivo aging of cells in the circulation, several different approaches were used. First, HPP spectrin content was correlated with red cell age by fractionating whole blood from HPP patients with a high reticulocyte count by Percoll density gradient centrifugation. Table 1 shows results of a representative experiment which indicated that the spectrin/band 3 ratio of young and old HPP Sp $\alpha^{I/46}$ red cells is similar to the ratio observed in whole blood. Similar results were obtained with HPP Sp $\alpha^{I/74}$ subjects, indicating that HPP cells of all ages are partially deficient in spectrin, suggesting that spectrin is not lost or degraded during circulating red cell maturation and that the deficiency is already manifested at the reticulocyte level.

Additional evidence for the absence of proteolytic degradation of spectrin in the circulating red cell was provided by immunoblot analysis using an affinity-purified antispectrin antibody. Figure 2 illustrates a representative SDS polyacrylamide gradient gel stained with Coomassie blue and the corresponding immunoblot of control and HPP Sp $\alpha^{I/46}$ whole cells, membranes, and cytosol. HPP Sp $\alpha^{I/74}$ subjects gave identical results (not shown). The immunoblot shows the presence of trace amounts of spectrin fragments in whole cells and membranes, visualized as faint bands below band 2, but these fragments are present in both control and HPP preparations. Furthermore, there is no evidence of spectrin or spectrin degradation products in the cytosol. These findings suggest that there is no abnormal proteolytic degradation of membrane-bound HPP spectrin, although they do not exclude the possibility that spectrin may be degraded into very small peptides not detected by the experimental procedure.

Spectrin content remains constant during in vitro red cell incubation. The HPP spectrin molecule is known to be thermally unstable[7]; during incubation at 37 °C some spectrin aggregation occurs, as evidenced by cross-linking experiments.[9] To ascertain whether this phenomenon of spectrin aggregation and denaturation contributes to the decreased spectrin content, possibly by rendering the molecule susceptible to proteolytic degradation, the spectrin content of incubated HPP red cells was examined. HPP Sp $\alpha^{I/74}$, as well as HPP Sp $\alpha^{I/46}$ cells, were incubated in vitro at 37 °C in a buffer that allowed them to maintain adequate ATP and GSH levels. Control cells in which the spectrin had been thermally denatured by heating to 49 °C for 20 minutes were also incubated under identical conditions. Table 2 shows the result of a typical experiment which indicated that the spectrin/band 3 ratio of incubated samples is the same as that found in comparable fresh cells, suggesting that spectrin denaturation does not induce degradation of the molecule and does not affect the spectrin content of the membrane.

Degradation of free HPP spectrin in erythroid precursor cells. The preceding experimental evidence indicates that HPP spectrin is stable on the membrane of the circulating red blood cell. However, nucleated erythroid precursor cells

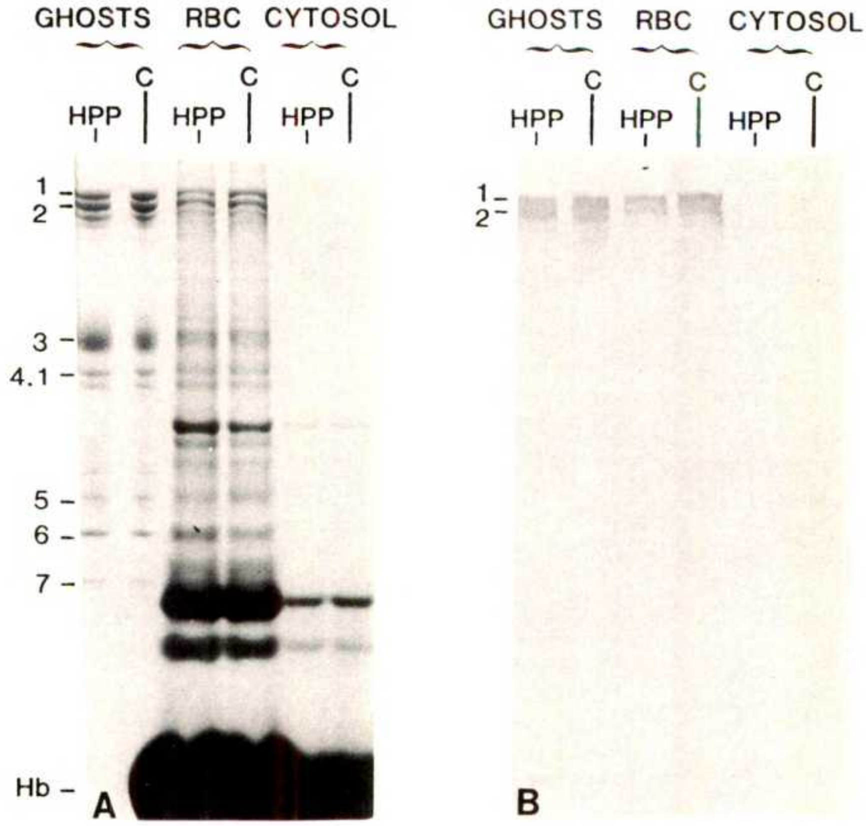

Fig 2. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and (B) immunoblots of ghosts, red blood cells, and cytosol. Proteins from control (C) and HPP Sp α I/46 ghosts, whole cells, and cytosol were subjected to SDS-PAGE on a 4.5% to 15% gradient polyacrylamide gel. Left, Coomassie blue-stained gel; right, corresponding immunoblot with affinity-purified antispectrin antibody. Trace amounts of spectrin are visualized on the immunoblot as a series of faint bands below band 2.

exhibit increased proteolytic activity[23]; therefore, the possibility that free cytosolic spectrin may be excessively degraded in bone marrow erythroid precursors, prior to its assembly on the membrane, was investigated. An in vitro experiment, using nucleated red cells from the spleens of anemic rats, was devised to test the susceptibility of free HPP spectrin to degradation by these proteases. Crude preparations of control and HPP spectrin were incubated with a lysate of nucleated red cells and, at various intervals, samples were analyzed by SDS-PAGE and immunoblotting techniques with either an affinity-purified antispectrin antibody or an antibody against the α I domain of spectrin.

Figure 3 shows representative Coomassie blue-stained gels and the corresponding immunoblots. Results showed that free HPP $\alpha^{I/46}$ spectrin, especially the α chain, was much more susceptible than control spectrin to proteolytic degradation from erythroid precursor cells. This is clearly seen in both the stained gels and the immunoblots with antispectrin and anti-α I antibodies. In contrast, free HPP $\alpha^{I/74}$ spectrin does not show any enhanced susceptibility toward degradation. Neither do purified HPP $\alpha^{I/74}$ spectrin dimers, which are enriched in the abnormal α I peptide, show any increased degradation when compared with purified control dimers (data not shown). These findings suggest that for HPP Sp $\alpha^{I/46}$ the decreased spectrin content may be partly due to increased proteolysis of free spectrin in erythroid precursor cells.

DISCUSSION

In this study, we demonstrated that red cell membranes of all 13 HPP patients studied in our laboratory were partially deficient in spectrin. The membrane spectrin content is reduced by ~30% in HPP individuals, as evidenced by a decreased spectrin/band 3 ratio. We demonstrated that this partial spectrin deficiency was not due to spectrin denaturation or proteolytic degradation in peripheral HPP red blood cells since the spectrin content did not correlate with cell age nor did it change during in vitro incubation of red cells. Furthermore, very few spectrin fragments (also present in

Table 1. Correlation of HPP Spectrin Content With Red Cell Age

Fraction No.	Reticulocytes (%)	Spectrin/Band 3 Ratio
Whole Blood	26	0.68
1	70	0.69
2	15	0.65
3	3	0.67

Results shown are representative of one of three experiments. Each value represents the mean of duplicate determinations.

Table 2. Spectrin Content During In Vitro Red Cell Incubation

	Spectrin/Band 3 Ratio	
	Fresh	Incubated (37 °C, 18 h)
Control	1.00	1.04
HPP	0.73	0.73
Heat denatured control (49 °C, 20 min)	0.95	1.00

Results shown are representative of one of three experiments. Each value represents the mean of duplicate determinations.

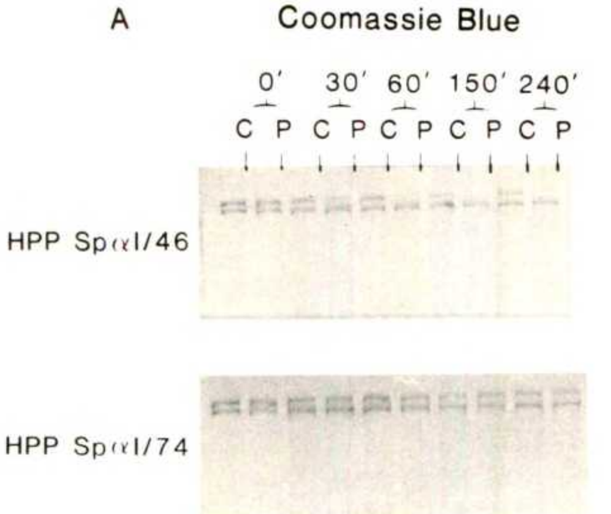

Fig 3. Degradation of spectrin by proteases of nucleated erythroid precursors. Crude spectrin from control (C), HPP Sp α I/74 (P), and HPP Sp α I/46 (P) subjects was incubated with a lysate of rat nucleated red cells for the intervals indicated. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. (A) Coomassie blue-stained SDS polyacrylamide gels; (B) immunoblots with affinity-purified antispectrin antibody; (C) immunoblot with anti α-I spectrin antibody.

control preparations) were detected in whole HPP cells or in isolated membranes, and no spectrin was present in the cytosol. These findings imply that membrane-bound spectrin is stable in circulating HPP red blood cells.

To determine whether spectrin might be prone to degradation in the bone marrow prior to the release of cells into the circulation, in vitro incubation experiments with nucleated red cell proteases were performed. Although incubation conditions did not necessarily represent the natural in vivo process, they did serve as an analogue of proteolytic conditions in the developing red cell. Results indicated that free HPP $\alpha^{I/46}$ spectrin was more susceptible than control spectrin to proteolytic degradation, suggesting that in these subjects the abnormal spectrin is degraded in erythroid precursor cells, probably prior to its assembly on the membrane; this could account for the observed partial deficiency in these individuals. In contrast, however, spectrin from HPP Sp $\alpha^{I/74}$ subjects was not excessively proteolysed when compared with control spectrin, indicating that the situation is more complex and that another mechanism contributes to spectrin deficiency in HPP. It is conceivable that an abnormality in spectrin synthesis is involved; HPP subjects may be heterozygotes for a mutant spectrin and for a variably expressed synthetic defect. This possibility is currently being evaluated in our laboratory.

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Aggressive Natural Killer Cell Leukemia in an Adult With Establishment of an NK Cell Line

By Louis A. Fernandez, Barbara Pope, Christine Lee, and Ekram Zayed

There have been many reports of cases in which chronic increases in the numbers of natural killer (NK) cells have been reported. Whether this is reactive or neoplastic in nature has been debated. We report the first case of an aggressive NK cell leukemia in an adult with establishment of an NK cell line. A 70-year-old man had two spontaneous episodes of jejunal perforation and one month later developed a severe febrile illness with moderate splenomegaly. Hemoglobin was 13.1 g/L, and WBC count was 1.8 × 10^9/L with 2% large granular lymphocytes (LGLs). Platelet count was 143 × 10^9/L; prothrombin time (PT) and partial thromboplastin time (PTT) were normal. Bone marrow was infiltrated with 25% to 30% LGLs; serum lysozyme was normal. Serum LDH was initially 1,191 U/L and rose to 6,408 (normal 240 to 525 U/L). Ten days later, the WBC count increased to 99.9 × 10^9/L with 70% LGL cells; the PT and PTT increased, and the platelet count dropped. No bacterial or viral cause of fever was identified. The cells from peripheral blood were LGLs that stained positively for acid phosphatase. All of the LGLs reacted with a monoclonal antibody reactive with NK cells (LEU-11b). Functionally, the patient's peripheral blood mononuclear cells (PBMs) demonstrated 100 times more lytic activity against K562 tumor cell lines than did normal PBMs. The patient's PBMs were propagated in vitro. The cultured cells showed the morphological, cytochemical, immunological, and functional characteristics of NK cells. In addition, partial trisomy involving chromosome 1q with duplication in regions of q21 through q31 was observed in all metaphases analyzed. The extra chromosome 1q with duplication in regions q21 through q31 was translocated to the p-terminal of chromosome 5. One percent to 5% of normal PBMs comprise NK cells; in most cases, leukemias arise from normal phenotypic counterparts. This case demonstrated that aggressive NK cell leukemia may occur in adults. In addition, the chromosomal abnormalities suggest that this is not a reactive process but a malignancy.

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GREAT HETEROGENEITY exists among human mononuclear cells, yet in almost all cases of leukemia, normal counterparts for the phenotypic patterns of leukemia cells have been identified.[1,2] The natural killer (NK) cell is one such cell type. Normally, NK cells comprise ~3.6% of peripheral blood mononuclear cells[3] and are also present in the bone marrow and spleen.[4] Morphologically, NK cells are large granular lymphocytes (LGLs), with cytoplasmic granules that stain positively for acid phosphatase[5] and react with monoclonal anti LEU-11b.[6] The cells are identified functionally by their ability to lyse specific targets, such as the K562 tumor cell.[4] Numerous case reports have described patients with long-standing increases in the numbers of NK cells; whether this increase is reactive or neoplastic in nature has been debated.[7-9] In addition, a few cases of NK cell leukemia in children from whom cell lines have been successfully established have been described.[10-11] We believe that this is the first report of an aggressive NK cell leukemia in an adult with the establishment of a leukemia cell line that retained the morphological, immunologic, and functional characteristics of NK cells.

Most lymphomas initially exhibit infiltration of organs by a particular mononuclear cell type. In many such cases, the mononuclear cell may suddenly increase in the peripheral blood and a leukemic state is heralded. We have observed a case in which the particular cell type was identified as a NK cell. Initially, leukemic cells were present in the bone marrow, spleen, and the lamina propria of the jejunum. Within ten days, the functionally active NK cells increased in the peripheral blood. The patient became febrile, developed a bleeding diathesis, and died.

MATERIALS AND METHODS

Patient. A 71-year-old white man who had an acute abdomen was found to have a jejunal perforation. At this time, an incidental abdominal aneurysm was noted. One month later, he again developed jejunal perforation. Two months later, he was admitted to the surgical ward for repair of his aortic aneurysm. He was febrile, with a temperature of 39 °C. He had minimal cervical lymphadenopathy and moderate splenomegaly. His hemoglobin was 13.1 g/L, hematocrit .404, WBC count 1.8 × 10^9/L with 1% metamyelocytes, 6% bands, 78% polymorphonuclear cells (PMNs), 13% lymphocytes, and 2% cells that had a morphology resembling LGLs. Platelet count was 143 × 10^9/L, prothrombin time (PT) was 11 seconds, and partial thromboplastin time (PTT) was 32.2 seconds. A bone marrow aspirate showed dysplastic erythropoiesis, but was infiltrated with 25% to 30% cells that had the morphology of LGLs. Serum lysozyme was 5.7 μg/mL (normal 3.3 to 6.6 μg/mL). Serum lactic dehydrogenase (LDH) initially was 1,191; later in the course of the disease, it rose to 6,408 U/L (normal 240 to 525 U/L). EKG and chest x-ray were normal. The ultrasound showed no intraabdominal collection of fluid. Liver and spleen scan showed a normal liver and an enlarged spleen. Protein electrophoresis was relatively normal, with IgG 13.42 g/L, IgA 4.79 g/L, and IgM 0.4 g/L. Antinuclear antibody and anti-DNA was within normal limits. Viral studies, which included hepatitis antigen and antibody, hepatitis surface antigen, toxoplasma, and EBV, were negative. Blood cultures for aerobic and anaerobic organisms were negative. Because the patient was relatively neutropenic, febrile, and constitutionally ill, he was treated empirically with carbenicillin, tobramycin, metronidazole, and prednisone.

One day after admission, he developed bleeding from his gastrointestinal tract. His platelet count was 15 × 10^9/L. Both PT and PTT were more than two minutes. White cell count was 35.4 × 10^9/L, with 50% large granular LGLs. At gastroscopy, multiple gastric ulcers ranging in size from 0.5 to 2 cm in diameter were seen. There was nodularity of the mucosa. He was treated symptomatically with H_2 blockers, transfusion of fresh frozen plasma, and cryoprecipitate

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without any change in the bleeding diathesis. Very rapidly, he developed generalized ecchymosis and died. Just before death, his white count had risen to 99.9 × 10^9/L, with 70% LGLs. Autopsy showed that he had infiltration of LGLs in the spleen, lymph nodes, and bone marrow. Autolytic changes made interpretation of the exact nature of infiltrating cells in the lamina propria and submucosa of the stomach and small bowel difficult. However, review of small bowel removed at initial surgery for perforation revealed that cells that were compatible with the morphology of LGL were infiltrating the lamina propria.

Morphology of cells. Peripheral blood and bone marrow slides and the cultured NK cell line were stained routinely with Wright's-Giemsa, peroxidase, Sudan Black B, periodic acid-Schiff (PAS), specific and nonspecific esterase and acid phosphatase. In addition, suspensions of PBMs and the cultured NK cell line were stained with fluorescein-conjugated monoclonal antibodies to OKT3, OKT4, OKT8, and LEU-11b antigens.[12] In brief, 5 μL of the reconstituted monoclonal antibody to OKT3, OKT4, OKT8 and LEU-11b antigens was added to 200 μL of cell suspension containing RPMI 1640 and 5% fetal calf serum (FCS). The cell suspension was mixed, placed on ice for 30 minutes, and mixed every ten minutes. The cell suspension was then washed twice with cold RPMI 1640 and resuspended in 100 μL of RPMI 1640. To this suspension was added 100 μL of fluorescein-conjugated goat anti-mouse antibody in 1:20 dilution. The suspension was mixed, placed on ice for 30 minutes, and mixed every ten minutes; it was then washed three times with cold phosphate-buffered saline (PBS). Two to three drops of buffered glycerol saline were added to the cell button, mixed and placed on a glass slide, and covered with a cover slip. Cells with fluorescence were enumerated by a Zeiss ultra violet microscope with a vertical illuminator.

In vitro establishment of NK cells. Heparinized blood from normal individuals or from our patient was diluted 1:1 with PBS and centrifuged for 30 minutes at 400 g on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). The mononuclear cell fraction remaining at the interface was washed with PBS and resuspended in complete medium [RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 2 mmol/L of glutamine, and 5 × 10^{-5} mol/L of 2-mercaptoethanol].

Mononuclear cells from the patient were cultured in 25-cm^2 flasks (Falcon #3021, Falcon, Oxnard, Calif) in 10 mL of complete medium at an initial concentration of 10^6 cells per milliliter. In some of the cultures, the medium was supplemented with 20% T cell growth factor (TCGF). The TCGF was cultured from the cell-free supernatant of cultured rat spleen cells which had been pulsed for two hours with 10 μg/mL Concanavalin A, washed three times with PBS, and incubated for 24 hours at 5 × 10^6 cells/mL in complete medium.[13] In all cases, approximately two-thirds of the medium was replaced in the cultures three times per week. To maintain cell numbers of ~10^5 cells per milliliter, cultures were subdivided when sufficient growth occurred.

Estimation of cytotoxic activity. Cytotoxicity was estimated by chromium release assay.[14] Approximately 2 × 10^6 K562 target cells were suspended in 0.2 mL of FBS and incubated for one hour at 37 °C with 100 μCi ^{51}Cr (NEN, Lachine, Quebec). Target cells were washed three times with PBS and resuspended in complete medium at 10^5 cells per milliliter. Target cells were pipetted in 0.1-mL vol into 96-well tissue culture multitrays (#76-013-05, Flow Laboratories, McLean, Va) to give 10^4 cells per well. The effector cells were suspended in complete medium at a range of concentrations and were pipetted into the trays in 0.1-mL vol to give a range of effector-target (ET) ratios. The trays were centrifuged for five minutes at 40 g, incubated for four hours at 37 °C in 5% CO_2, and centrifuged for ten minutes at 700 g. The supernatant was removed from the cells, and 100-μL samples were counted in a Beckman Gamma 8000 counter. Maximum release was estimated as cpm from samples containing 1% Triton X-100. Minimum release was estimated in cultures containing medium alone. The percentage of lysis of the target was calculated as: sample CPM − minimum CPM/ maximum CPM − minimum CPM × 100.

Chromosome analysis. Cells cultured in TCGF-supplemented medium were harvested on the tenth day. Metaphase spreads were prepared by the conventional air-dry technique and stained according to G-banding procedures.[15]

RESULTS

Characterization of LGLs from peripheral blood, bone marrow, and established cell line. Peripheral blood LGLs were variable in size. Most of the LGLs had a low nuclear-cytoplasmic ratio and intense basophilic cytoplasm that contained prominent azurophilic granules. Relatively dense chromatin and nuclei were observed in most of these cases. Auer rods were absent, and rare vacuoles were detected (Fig 1). LGLs stained positively for both acid phosphatase and nonspecific esterase. LGLs did not express OKT3, OKT4, or OKT8 surface antigens. However, 100% of these cells stained with LEU-11b monoclonal antibody.

The cellularity of the bone marrow was moderately increased, with megaloblastic and dysplastic erythropoiesis. Granulopoiesis and megakaryocytopoiesis generally appeared normal. The pleomorphic mononuclear cells constituted 25% to 30% of all nucleated cells in the marrow. The morphology was the same as that of those seen in the peripheral blood (Fig 2). Cytochemical reactions were not conclusive; however, the LGLs stained positively for both acid phosphatase (Fig 3) and nonspecific esterase.

The cells from the established cell line had morphology and cytochemistry similar to that of the peripheral blood and bone marrow LGLs. In addition, the established cell line did not stain with OKT3, OKT4 or OKT8 antibodies. However, all of these cells stained with LEU 11b monoclonal antibody, similar to the immunologic characteristics of the peripheral blood LGL.

Growth of NK cells. PBMs from our patient maintained viability in culture for two weeks. During this time, there was no apparent difference between cultures maintained with or without growth factors. However, in the third week the cells entered a "crisis period." Cells cultured without TCGF died,

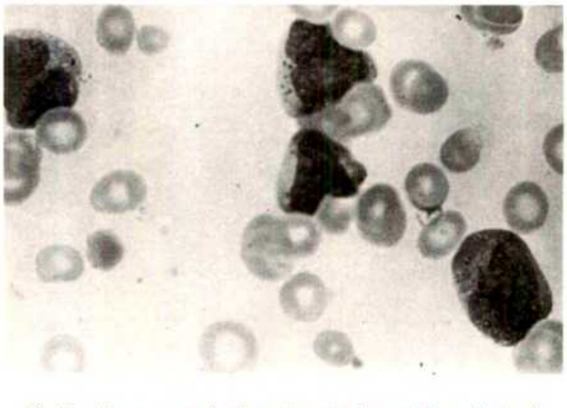

Fig 1. Large granular lymphocytes in peripheral blood.

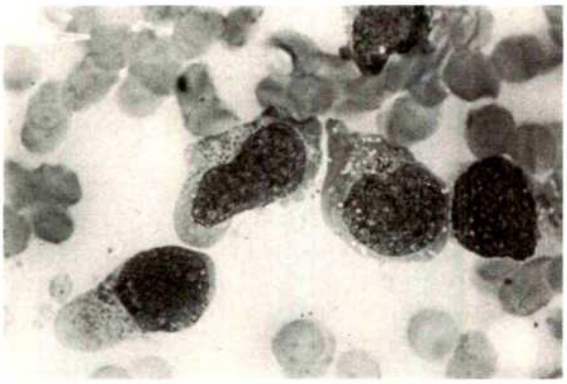

Fig 2. Large granular lymphocytes in bone marrow.

Table 1. Cytotoxic Activity of NK Cells

Sample Tested	Source of Cells*	Lysis of K562 (%) at ET Ratio†				
		100:1	25:1	4:1	1:1	.25:1
Normal 1	PBMs	48.5	27.4	—	—	—
Normal 2	PBMs	46.6	13.6	—	—	—
Normal 3	PBMs	19.8	8.2	—	—	—
Patient	PBMs	90.1	89.6	63.1	35.5	11.6
	9-Day culture (no TCGF)	88.4	86.5	63.8	26.5	—
	9-Day culture (TCGF)	85.4	85.5	54.1	21.7	—
	35-Day culture (TCGF)	—	—	77.3	45.7	20.5
	60-Day culture (TCGF)	—	—	95.2	43.8	17.8

*Fresh peripheral blood mononuclear cells (PBM) or PBMs from the NK cell leukemia patient cultured with or without growth factor (TCGF) for varying lengths of time.

†Varying numbers of effector cells were mixed with 10^4 K562 target cells to give the effector-target (ET) ratios.

and cells cultured with TCGF did not proliferate. After an additional two weeks of maintenance, cells cultured in TCGF began to proliferate and were maintained for an additional two months in culture. Sufficient growth was obtained to subculture twice a week (1:3 split).

Cytotoxic assays. Table 1 shows the results of assays of the cytotoxic activity of fresh PBMs from three normal individuals and from the patient. The cytotoxic activity of PBMs of the patient was considerably higher than that seen in normal individuals. The activity of fresh PBMs from the patient at an ET ratio of 1:1 was equivalent to that seen at a ratio of 100:1 in some normal individuals. In addition, this ability to lyse K562 target cells was maintained when the patient's PBMs were cultured, and good lytic activity was seen after 60 days in culture with TCGF.

Chromosomal analysis. Twenty cells were analyzed. Partial trisomy involving chromosome 1q with duplication in regions q21 through q31 was observed in all 20 metaphases. The extra chromosome 1q with duplications in regions q21 through q31 was translocated to the p-terminal of chromosome 5 (Fig 4). The absence of the Y chromosome in 9 of 20 cells (45%) analyzed was also significant.

DISCUSSION

In this article, we provide evidence in an adult for a case of highly aggressive leukemia with the morphology and functional capability of NK cells. This conclusion was based on the finding, in the patient's blood and marrow, of abnormally high numbers of LEU-11b, $T3^-$, $T4^-$, $T8^-$, LGLs that stained positively for acid phosphatase. The conclusion that the leukemia cells were NK cells was strengthened by the finding that the patient's PBMs had a greatly enhanced ability to lyse K562 target cells. That these abnormal LGLs were malignant, rather than the result of an abnormal proliferation of NK cells, was based on the finding of chromosomal abnormalities in a cell line established from the patient's peripheral blood.

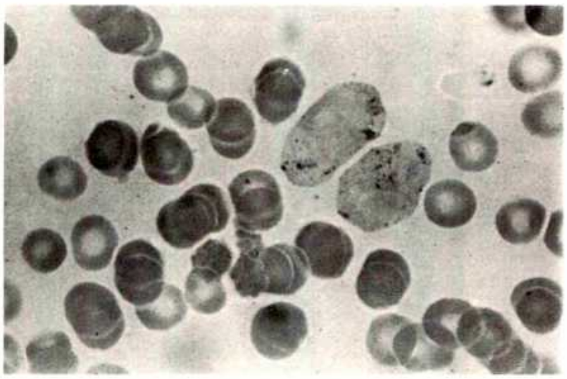

Fig 3. Acid phosphatase staining of large granular lymphocytes in peripheral blood.

In normal individuals, most NK cells are present in the peripheral blood, bone marrow, and spleen. In contrast, NK cells are rare in the lymph nodes. In animals, NK cells have also been found in the lamina propria of the small bowel.[16] Our patient initially developed jejunal perforation in a location where NK cells have been found experimentally. In addition, he had splenomegaly without lymphadenopathy; 30% of his marrow cells and, ultimately, 70% of his PBMs were LGLs.

The patient was mildly granulocytic. The cause is unknown; however, this effect may have been due to the suppressive effect of the leukemia cells. It is known that NK cells suppress granulopoiesis in vitro.[17] Most patients with chronic NK cell leukemia develop severe neutropenia, recurrent infection, and hypogammaglobulinemia. However, our patient had neutropenia without evidence of recurrent infection.

The patient developed marked swings in temperature, but no cause of infection could be found. It is now known that NK cells produce Interleukin-1, which is analogous to endogenous pyrogen.[18] Thus, the febrile response may have been due to mediator release by the NK cells, although a viral cause cannot be excluded since enhanced NK cell activity had been observed during several acute viral infections.[19,20] Although the rise in NK cells in our patient may have been secondary to a viral infection, we believe that this is unlikely because of the observed combination of high levels of LGLs

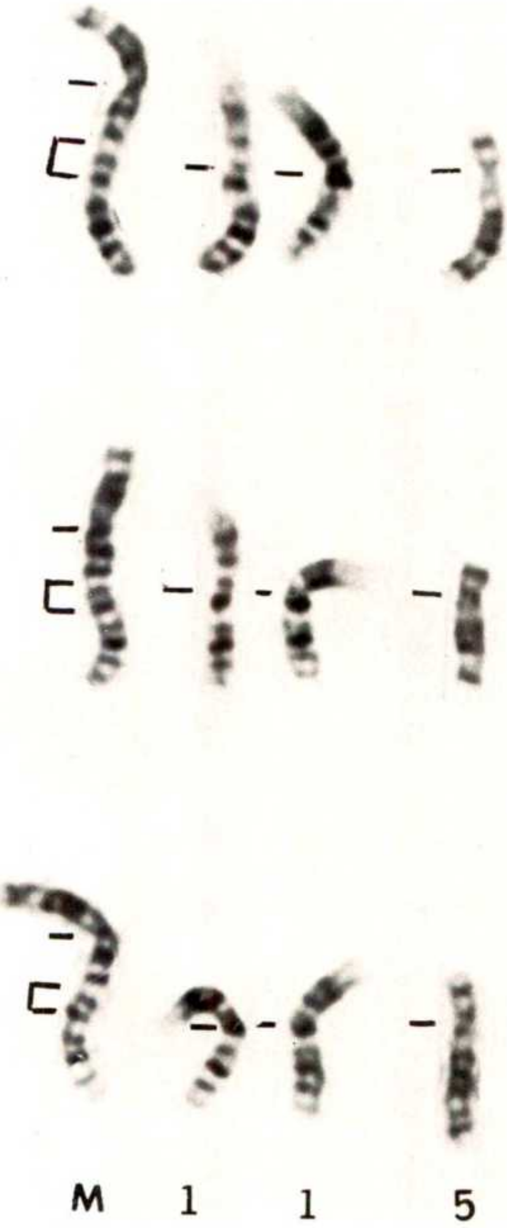

Fig 4. Partial karyotypes of three metaphases showing the marker chromosome (M), a pair of normal chromosomes (1) and a normal chromosome 5. The marker chromosome consists of chromosome 1q with duplication in bands q21 through q31 (bracket), translocated to the terminal region of the p-arm of chromosome 5. The centromere of each chromosome is indicated by a horizontal line. To compare the band pattern of the extra chromosomal material with the normal chromosomes 1q, the centromere of the marker chromosome is not aligned with the centromeres of the normal chromosomes.

in the patient's blood and marrow, enhanced lytic activity, and chromosomal abnormalities.

The coagulopathy that developed was suggestive of disseminated intravascular coagulation (DIC). DIC has been reported to occur in many malignant states, including leukemias, and is most common in acute promyelocytic leukemia.[21] However, in a few reported cases of increased NK cell activity representing either leukemia or lymphoma, DIC has not been reported.[7-11] Most cases of NK cell leukemia in adults are chronic in nature, and the disease is generally not aggressive.[22] Our case differed in its acute aggressive clinical course; in addition, our case did not express T cell antigens.

Cells with LGL morphology, with or without cytotoxic activity, have been described in patients with lymphoproliferative disorders,[8,9] in adults with chronic leukemia,[7] and in children with acute leukemias.[10,11] In some but not all cases, LGL morphology has been associated with the functional ability of the cells to lyse or bind to K562 target cells. In one case of acute leukemia, lytic activity was seen only after the incubation of the PBMs for 24 hours at 37 °C.[10,11] The PBMs from our patient not only had a high proportion of LGLs, but also displayed high, spontaneous cytotoxic activity at very low ET ratios. These results contrast markedly with those seen with PBMs from patients with lymphocytic leukemias of non-LGL morphology, in which NK cell activity has frequently been found to be depressed as compared with that of normal subjects.

Cell lines established from a child with lymphoblastic leukemia with NK activity have been previously reported.[10] These cell lines retained the surface markers and morphology of the leukemia cells, but lost much of their functional capability, having less lytic activity than that of normal PBMs. In our case, the established cell line retained the morphology, surface markers, and high cytotoxic activity of the leukemia cells seen in the fresh sample of blood from our patient. However, in both cases, the established cell lines were negative for T cell-associated surface markers and were positive for NK cell markers. The cell lines from the childhood leukemia case were negative for T3, T4, and T8, but were positive for Asialo-GM1. Both the fresh blood cells and the cell line from our patient were also negative for T3, T4, and T8, but were positive for the NK-associated marker, LEU-11b. This pattern of expression of surface markers is markedly different from that seen with most adult chronic leukemias and lymphoproliferative disorders expressing LGL morphology and NK cell activity.[7-9,22] These cells are uniformly positive for T3 and are frequently positive for T4 and/or T8. In addition, it is unlikely that the established cell line was an NK-like cell derived from a normal cell component of the patient's blood as has been reported by Brooks et al.[23] NK-like clones have been obtained from various sources including cultured cells, mixed leukocyte reactions, and cytotoxic T cell lines. However, such cell lines invariably expressed T cell-associated surface markers, even when the precursor cells were depleted of T cells prior to culture. Because our cell line never expressed T cell markers, and because the functional capability of the cells did not alter upon culture, it is most likely that the cultured cells were derived from the leukemic LGLs in the patient's blood.

Essentially all human NK cell activity is mediated by LEU-11$^+$ and LEU-7$^+$ cells although LEU-11$^+$, LEU-7$^-$ cells have the most potent lytic activity and LEU-11$^-$, LEU-7$^+$ cells have the weakest activity on a per cell basis.[6] The very potent activity seen with the cells of our patient was associated with LEU-11$^+$ cells.

Cytogenetic analysis of the cell line provided evidence that the LGL proliferation seen in this patient was a neoplastic rather than a reactive process. Cytogenetic analysis showed the presence of an extra chromosome, 1q, with duplication in regions q21 through q31 in all 20 metaphases analyzed. Although chromosomal abnormalities have been reported to arise spontaneously in vitro, they usually occur after 18 to 20 months of in vitro cell culture.[24] In our case, a 100% abnormal cell expansion of metaphases analyzed could not have arisen within ten days of culture before the cell crises occurred. Therefore, the established NK cell line was most likely derived from the leukemic NK cells in the patient. Clonal chromosomal abnormality involving the acquisition of an extra chromosome 8 or 14 was recently reported in two cases of chronic NK cell leukemia.[25] Our study documents clonal proliferation in a case of aggressive NK cell leukemia.

Partial trisomy involving the whole or part of the q-arm of chromosome 1, and in particular the duplication of regions q21 through q31, has been reported in a large variety of peripheral blood malignancies as well as in solid tumors.[26–28] Although an etiological role of this type of defect in human cancer cannot be ruled out completely, the general consensus was that abnormality involving chromosome 1q was a secondary event that was selected because it was able to confer proliferative advantage to the existing tumor cells.[29,30] Additional karyotypic changes involving the loss of a sex chromosome were observed in ~50% of myeloproliferative disorders.[31] Patients who had this additional abnormality seemed to have a poorer prognosis.[32] Almost half of the NK tumor-cloned cell population in our patient had also lost a Y chromosome.

In summary, our case demonstrates that aggressive NK cell leukemia ($T3^-$, $T8^-$, $T4^-$ and LEU $11b^+$) can develop in adults, probably arising from its normal phenotypic counterpart. These cells were established in vitro and retained their morphological, immunologic, and functional characteristics. The presence of chromosomal abnormalities suggests that this was not a reactive process but a malignancy.

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Modulation of Neutrophil Oxidative Responses to Soluble Stimuli by Platelet-Activating Factor

By James C. Gay, Jeffrey K. Beckman, Karen A. Zaboy, and John N. Lukens

The role of platelet activating factor (PAF) as a regulator of human neutrophil superoxide (O_2^-) generation in response to soluble and particulate stimuli was examined. At concentrations $>10^{-7}$ mol/L, PAF alone induced a brief burst of O_2^- production. When cells were exposed to PAF and either the chemotactic peptide *n*-formyl-methionyl-leucyl-phenylalanine (FMLP 10^{-7} mol/L) or the tumor promoter phorbol myristate actate (PMA 10 ng/mL), a marked synergistic augmentation of O_2^- release was noted when compared to control cells stimulated with FMLP or PMA alone. Mean percentage of enhancement by 10^{-5} mol/L of PAF was 297% ± 35% (n = 9) of control responses to FMLP and 185% ± 16% (n = 3) of control responses to PMA. Consistent enhancement occurred with PAF concentrations of as low as 10^{-9} mol/L. Enhancement could be demonstrated when neutrophils were exposed to PAF either at the same time as, or up to 60 minutes prior to, the second stimulus, and was neither reversed by removal of PAF from the medium prior to addition of FMLP or PMA nor dependent on the presence of extracellular divalent cations. Continuous recordings revealed that the enhancement was due to an increased maximal rate of O_2^- production. In contrast, PAF concentrations up to 10^{-5} mol/L had only a minimal effect on the response to neutrophils to opsonized zymosan. Analysis of the enhancing properties of lipids structurally related to PAF revealed that the critical moiety was the saturated fatty acid at position 1. These results indicate the presence of a PAF-mediated positive feedback loop whereby the oxidative burst induced by some soluble stimuli is augmented. Modulation of neutrophil O_2^- production by PAF may serve to amplify neutrophil oxidative responses at sites of inflammation.

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ACETYL GLYCERYL ether phosphorylcholine (platelet-activating factor, PAF) is a recently described lipid that is a potent activator of both platelets and neutrophils. PAF stimulates neutrophil degranulation, aggregation, oxidative metabolism, and chemotaxis.[1-5] In addition to having direct effects on neutrophils, PAF has been shown to modulate cellular responses. For example, preincubation of neutrophils with PAF causes inhibition of subsequent aggregation in response to leukotriene B_4 and to PAF itself.[6,7]

Other neutrophil stimuli, especially chemotactic factors, also modify the cell's response to other agents. For example, the chemotactic peptides formyl-methionyl-leucyl-phenylalanine (FMLP) and C5a enhance neutrophil superoxide (O_2^-) production in response to the nonchemotactic agents phorbol myristate acetate (PMA) and fluoride ion,[8] whereas they inhibit subsequent responses to higher concentrations of homologous peptides.[9] We have recently shown that leukotriene B_4 (LTB_4), a potent lipid chemoattractant and weak O_2^- agonist itself, markedly enhances the ability of neutrophils to release O_2^- in response to FMLP but does not alter oxidative responses to PMA or zymosan.[10]

PAF has potential for modulating neutrophil oxidative repsonses for several reasons. First, other agents that effect neutrophil aggregation[11] also modulate oxidative responses.[9,10] Second, like other known chemotactic factors, its chemotactic properties suggest that PAF may regulate neutrophil functions. Third, PAF elicits the production of LTB_4 by neutrophils,[12,13] and some investigators have suggested that many of the effects of PAF on neutrophils are mediated by this leukotriene.[12] Because LTB_4 enhances O_2^- production induced by FMLP,[10] PAF may possess similar properties. We have therefore studied PAF as a regulator of neutrophil O_2^- production in response to other agents. Our results indicate that PAF is a potent enhancer of neutrophil oxidative responses to soluble stimuli. These observations suggest the presence of yet another lipid-mediated positive feedback loop that may contribute to the oxidative explosion that occurs when neutrophils arrive at sites of inflammation.

MATERIALS AND METHODS

Ferricytochrome *c* (type III), zymosan, dimethyl sulfoxide, cytochalasin B, FMLP, Ficoll-Hypaque (Histopaque), superoxide dismutase (SOD), PMA, bovine serum albumin (BSA), and L-α-phosphatidylcholine, β-acetyl-γ-O-alkyl (PAF), DL-α-lysophosphatidylcholine, γ-O-hexadecyl (lyso-PAF), L-α-lysophosphatidylcholine, palmitoyl (lyso-PC), L-α-glycero-phosphorylcholine (GPC), diolein, 1-oleoyl, 2-acetyl glycerol, and 1-monopalmitoyl-rac-glycerol (monopalmitin) were purchased from Sigma Chemical Company, St Louis. Hanks' balanced salt solution (HBSS with or without calcium and magnesium) was obtained from GIBCO, Grand Island, NY. Eicosatetraynoic acid (ETYA) was a generous gift from Hoffman-La Roche, Nutley, NJ.

FMLP and PMA were dissolved in dimethyl sulfoxide at a concentration of 10 mg/mL These were further diluted in HBSS prior to use. Cytochrome *c* was dissolved in saline at a concentration of 0.8 mmol/L. SOD was dissolved in saline at a concentration of 1 mg/mL.

Zymosan was opsonized by incubation in fresh normal serum (10 mg/mL) at 37 °C for 30 minutes. The particles were then washed by centrifugation and resuspended in HBSS at a concentration of 5 mg/mL.

PAF and other lipids were stored at −4 °C in chloroform or ethanol. At the time of usage in neutrophil assays, an aliquot from the stock solution was evaporated under N_2 and redissolved in 2 5% BSA in normal saline prior to addition to the final incubation medium.

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Neutrophil suspensions. Human neutrophils were isolated from heparinized venous blood from healthy adult volunteers by Ficoll-Hypaque gradient centrifugation, as previously described.[9]

Neutrophil superoxide release Superoxide (O_2^-) was assayed by measuring the O_2^--dependent (SOD-inhibitable) reduction of ferricytochrome *c*, as previously described.[14] Assays were performed in both batch and continuous fashion. All experiments were performed in triplicate and were repeated at least twice for verification. For batch experiments in which O_2^- production was measured at a single time, a suspension of neutrophils (1.5×10^6 cells) was incubated at 37 °C for ten minutes, after which PAF (or other lipid) was added to cells at the indicated concentration. Cells were then exposed to HBSS or an oxidative stimulus (FMLP, PMA, or opsonized zymosan). Incubations were continued for 5 minutes with FMLP, 10 minutes with PMA, and 10 minutes with zymosan before measurable cytochrome *c* reduction was halted by the addition of 20 μg/mL of SOD to all reaction tubes. The extent of cytochrome *c* reduction in each supernatant was determined by the change in absorbance at 550 nm (Gilford 250 recording spectrophotometer, Gilford Instrument Laboratories, Oberlin, Ohio) in reference to blanks to which SOD was added prior to any oxidative stimulus. Superoxide concentrations were calculated by means of the extinction coefficient 15.5/mmol/L per centimeter of ferricytochrome *c*[14] Results obtained in the presence of stated lipid concentrations are expressed as either nmols O_2^- per $1 5 \times 10^6$ polymorphonuclear leukocytes (PMNs) or percentage of response obtained in the absence of the lipid. Control neutrophil suspensions were preincubated with equal volumes of 2.5% BSA prior to stimulation. For experiments dealing with the influence of divalent cations on the effects of PAF, neutrophils were maintained in calcium-free and magnesium-free buffers. Indicated concentrations of these cations were added back to some reactions tubes prior to addition of PAF or its solvent.

Continuous recordings of O_2^- production were performed to assess the effects of PAF on the kinetics of O_2^- generation in response to various oxidative stimuli as previously described[15] using a Gilford 250 recording spectrophotometer. Activation time, maximal velocity (V_{max}), and cumulative absorbance at a given time were measured from the curves obtained.

Statistics. Variance is expressed as mean ± SE.

RESULTS

PAF-induced O_2^- production. As reported by others,[1,2] PAF is a direct stimulus of neutrophil O_2^- generation (Fig 1A). However, at the cell concentrations used in these studies (1.5×10^6 PMNs), only a small amount of O_2^- was released in response to PAF and only at concentrations $>10^{-6}$ mol/L. The direct response to PAF was decreased to 68% of control values when calcium and magnesium were omitted from the suspending medium (data not shown).

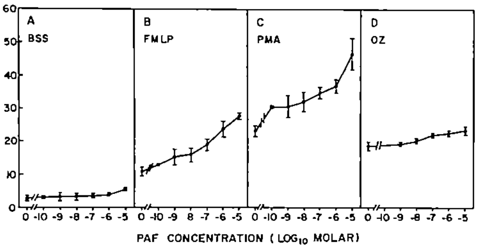

Fig 1. Effect of platelet-activating factor (PAF) on neutrophil O_2^- production. Cells were exposed to the indicated concentration of PAF immediately prior to addition of buffer or stimulus and incubation at 37 °C. (A) Hank's balanced salt solution (HBSS) for 10 minutes; (B) formyl-methionyl-leucyl-phenylalanine (FMLP), 10^{-7} mol/L for 5 minutes; (C) phorbol myristate acetate (PMA), 10 ng/mL for 10 minutes; (D) opsonized zymosan (OZ), 250 μg/mL for 10 minutes. Data points represent mean ± SE of experiments using cells from at least two donors.

Effects of PAF on neutrophil responses to other oxidative stimuli. Also illustrated in Fig 1 is the effect of PAF on neutrophil oxidative responses to FMLP, PMA, and opsonized zymosan. Responses to FMLP and PMA (Fig 1B and C) were consistently enhanced by PAF in concentration-dependent fashion. At the highest PAF concentration used (10^{-5} mol/L), enhancement averaged 297% ± 35% (n = 9) of controls for FMLP (10^{-7} mol/L) and 185% ± 16% (n = 3) of controls for PMA (10 ng/mL). Consistent slight enhancement was evident at PAF concentrations as low as 10^{-9} mol/L (143% ± 12% of controls with FMLP and 126% ± 7% of controls for PMA). The concentration of FMLP used in these experiments (10^{-7} mol/L) represents an optimal oxidative concentration of the peptide. In the presence of higher PMA concentrations, the percentage of enhancement by PAF decreased as the control response to PMA increased such that no enhancement could be demonstrated with a PMA concentration of 3.2×10^{-7} mol/L (200 ng/mL; Table 1.) Maximal absolute increase in O_2^- production seen in the presence of PAF was noted at a PMA concentration of 10 ng/mL, although the percentage of increase was greater at lower PMA concentrations. PAF, therefore, was a more potent enhancer at submaximal concentrations of the phorbol ester. The effect of PAF on the O_2^- response to FMLP and low concentrations of PMA was synergistic rather than additive. In contrast, PAF concentrations up to 10^{-5} mol/L had only a minimal enhancing effect on the response to zymosan (250 μg/mL) (Fig 1D).

Characteristics of PAF-enhanced responses. PAF enhancement of the neutrophil oxidative response to FMLP did not require incubation of neutrophils with the lipid prior to exposure to the peptide (Table 2). In our experience and that of other investigators,[1] the duration of O_2^- production in

Table 1. PAF Enhancement of Oxidative Responses to Varying PMA Concentrations

PMA Concentration (ng/mL)	PAF (10^{-8} mol/L)	O_2^- Production* (% Control)
200	–	57.46 ± 0.43
200	+	57.70 ± 0.39 (100)
20	–	54 34 ± 0.15
20	+	57.46 ± 0.40 (106)
10	–	32.29 ± 0 23
10	+	54.71 ± 1.23 (169)
5	–	14 30 ± 0.32
5	+	32.39 ± 0.50 (227)
2	–	3 74 ± 0.16
2	+	10 65 ± 0.08 (285)

PAF, platelet-activating factor; PMA, phorbol myristate acetate.

Neutrophils were simultaneously exposed to buffer(–) or PAF(+) and the indicated concentration of PMA for ten minutes at 37 °C

*Neutrophils (nmols O_2^- per 1.5×10^6), mean ± SE of triplicate determinations from a single representative experiment.

Table 2. Effect of Preincubation Time on PAF-Mediated Enhancement of FMLP-Induced O_2^- Generation

Preincubation Time (min)*	O_2^- Production† (% Control Response)
0	52.45 ± 0.36 (203)
1	52.90 ± 1.60 (204)
5	56.60 ± 0.15 (219)
10	50.11 ± 0.67 (194)
20	48.34 ± 1.05 (187)
30	47.12 ± 1.85 (182)
40	45.48 ± 0.94 (176)
60	37.67 ± 0.81 (146)
FMLP alone	25.87 ± 0.68 (100)

PAF, platelet-activating factor; FMLP, formyl-methionyl-leucyl-phenylalanine

*Neutrophils (1.5×10^6) were exposed to PAF (10^{-6} mol/L) at 37 °C for 0 to 60 minutes prior to stimulation with FMLP (10^{-7} mol/L) for 5 minutes.

†Neutrophils (nmols O_2^- per 1.5×10^6); mean ± SE of triplicate determinations in a single representative experiment.

Table 3. Effect of Incubation Conditions on PAF Enhancement

Incubation Conditions	Neutrophils (nmols $O_2^-/1.5 \times 10^6$)
Buffer + FMLP*	9.14 ± 0.34†
PAF + FMLP	15.57 ± 0.32
Buffer + FMLP (without Ca^{2+}, Mg^{2+})	5.63 ± 0.18
PAF + FMLP (without Ca^{2+}, Mg^{2+})	10.02 ± 0.14
Buffer + PMA*	19.64 ± 0.52
PAF + PMA	50.35 ± 0.73
Buffer + PMA (without Ca^{2+}, Mg^{2+})	16.48 ± 0.21
PAF + PMA (without Ca^{2+}, Mg^{2+})	36.16 ± 0.65
Buffer + cytochalasin B + FMLP‡	24.54 ± 0.04
PAF + cytochalasin B + FMLP	32.95 ± 1.63
Buffer + FMLP§	15.44 ± 0.53
PAF (washed) + FMLP	32.34 ± 0.52
Buffer + PMA	18.80 ± 0.42
PAF (washed) + PMA	35.40 ± 0.83

PAF, platelet-activating factor; FMLP, formyl-methionyl-leucyl-phenylalanine, PMA, phorbolmyristate acetate.

*Neutrophils treated with buffer or PAF (10^{-6} mol/L) were exposed to 10^{-7} mol/L of FMLP for five minutes or PMA (2 ng/mL) for ten minutes in the presence of extracellular Ca^{2+} (1.3 mmol/L) and Mg^{2+} (0.5 mmol/L) or in the absence of the cations

†Mean ± SE of triplicate determinations.

‡Cells were exposed to cytochalasin B (5 μg/mL) for two minutes prior to addition of buffer or PAF (10^{-6} mol/L) and FMLP (10^{-7} mol/L).

§Neutrophils were incubated at 37 °C for two minutes with either buffer or PAF (10^{-6} mol/L) prior to centrifugation and two cell washings Cells were then resuspended in Hank's balanced salt solution with cytochrome *c* and exposed to FMLP (10^{-7} mol/L) for five minutes or PMA (2 ng/mL) for ten minutes at 37 °C.

response to PAF alone is brief. To determine whether the enhancing effect of PAF could be demonstrated after neutrophils had reached the plateau phase of response to PAF alone, cells were incubated with PAF for periods up to 60 minutes prior to addition of FMLP. As shown in Table 2, neutrophils exposed to PAF retained an enhanced responsiveness to FMLP even after 60 minutes of preincubation, although the effect tended to diminish with longer incubation times. Thus, PAF not only had synergistic effects with FMLP, but its enhancing effect persisted after the direct stimulating effect of PAF had reached completion. Similar effects were noted when PMA was used in place of FMLP as the secondary stimulus (data not shown).

Because we had demonstrated that optimal direct stimulation of O_2^- production by PAF was dependent on extracellular calcium, we sought to determine the calcium requirement for PAF enhancement of the response to FMLP and PMA. As noted by other investigators,[17] the response of neutrophils to FMLP was decreased in the absence of extracellular calcium (Table 3). However, in the absence of calcium, PAF-treated cells still exhibited enhanced responsiveness to the peptide, in a degree similar to enhancement seen in cells maintained in calcium-containing buffer. Enhanced responses were also noted when cells were exposed to PMA despite the absence of extracellular cations. Therefore, augmentation of FMLP-stimulated and PMA-stimulated O_2^- production by PAF did not require the presence of the divalent cation.

It is well known that the fungal metabolite cytochalasin B enhances measurable O_2^- generation in response to chemotactic peptides. The mechanism for this effect is not entirely clear but may be related to failure to internalize O_2^- produced because of impaired microfilament assembly or to aggregation or uncovering of peptide receptors.[18] Despite their already enhanced responsiveness to FMLP in the presence of cytochalasin B, neutrophils generated even greater amounts of O_2^- anion when simultaneously exposed to PAF (Table 3).

To determine whether the cellular response to PAF that resulted in enhanced responsiveness required the continued presence of PAF in the medium, neutrophils were exposed to PAF and washed twice prior to stimulation with FMLP or PMA. After being washed, PAF-treated cells continued to exhibit greater O_2^- production than did buffer-treated controls (Table 3).

Because other investigators have shown that PAF induces LTB_4 production in neutrophils,[12,13] we sought to determine whether the enhancing effect of PAF was dependent on formation of the leukotriene. As previously demonstrated[19] 10 μmol/L of ETYA, a lipoxygenase inhibitor, inhibited the response to FMLP by 59% ± 1% [FMLP alone = 16.54 ± 0.50 nmols O_2^- (n = 2); ETYA + FMLP = 6.77 ± 0.19 nmols O_2^- (n = 2)]. Despite the presence of the lipoxygenase inhibitor, PAF continued to enhance oxidative responses to FMLP (ETYA + PAF + FMLP = 33.23 ± 0.90 nmols O_2^-). Similarly, the presence of ETYA did not alter PAF enhancement of responses to PMA (data not shown). Thus, it is unlikely that the enhancing effect of PAF on neutrophils is mediated by LTB_4.

Effect of PAF on the kinetics of O_2^- production. We next examined the effects of PAF on the kinetics of the oxidative response by the continuous recording method of Cohen and Chovaniec.[15] The neutrophil oxidative response to the chemotactic peptide is characterized by a three- to six-second lag phase before O_2^- production is detected. This is followed by a progressive increase in O_2^- release which terminates three to four minutes after onset. The addition of PAF prior to addition of FMLP altered neither the activation time nor the total period of time during which the O_2^- forming oxidase was active (Fig 2). PAF-treated cells exhibited a markedly enhanced rate of O_2^- production, however, with a mean maximal rate (V_{max}) 205% ± 42% of control (n = 3). Thus, a markedly enhanced rate of O_2^- generation accounted for the augmentation of cumulative O_2^- measurements in FMLP-stimulated cells. An increase in V_{max} could be demonstrated even when PAF was added after FMLP. When PAF was added one minute after FMLP, during the time when the rate of O_2^- production is constant, the subsequent rate was increased although the total time of oxidase activity was unchanged. When PAF was added to cells two minutes after FMLP, when O_2^- production is beginning to diminish, an immediate increase in V_{max} still occurred, although the magnitude of the increase was less than when PAF was added earlier.

PAF also had striking effects on the kinetics of the response to PMA. The cellular response to PMA is characterized by a lag period prior to the onset of measurable O_2^- generation which is dependent on the PMA concentration. Figure 3 shows that when PAF was added simultaneously with PMA, the lag time was drastically shortened and the rate of O_2^- production increased markedly (mean percentage of enhancement 152% ± 11%, n = 7). When PAF was added two minutes prior to PMA, the lag time was unchanged (data not shown) although the V_{max} to O_2^- generation remained enhanced. It appears likely that the shortened activation time seen when both agents were added simultaneously is due to the direct effect of PAF, since the lag time in response to this agent is very short. When PMA was added after the direct effect of PAF was completed, the contribution of PAF to the shortened lag time was not seen. Even after preincubation of up to 20 minutes with PAF, an enhanced rate of O_2^- production was seen after addition of PMA.

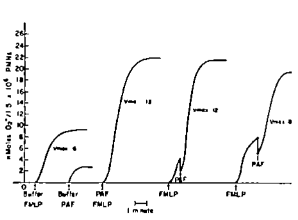

Fig 2. Continuous assays of O_2^- production in response to platelet-activating factor (PAF) and formyl-methionyl-leucyl-phenylalanine (FMLP). Neutrophils exposed to FMLP (10^{-7} mol/L) or PAF (10^{-6} mol/L) alone exhibited a brief burst of O_2^- production, which terminated after several minutes When PAF and FMLP were added simultaneously, a marked increase in rate of O_2^- production (V_{max}) was noted. A similar increase in V_{max} was noted even when PAF was added several minutes prior to FMLP (data not shown). Enhancement of V_{max} was demonstrable immediately when PAF was added either one or two minutes after FMLP. Curves represent assays performed using cells from a single donor. Similar results were obtained using cells from two different donors. V_{max}, maximal rate of O_2^- production (nmols O_2^-/min).

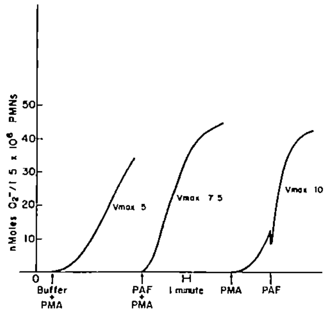

Fig 3. Platelet-activating factor (PAF) modifies the kinetics of O_2^- generation in response to phorbol myristate acetate (PMA). Neutrophils exposed to PMA (10 ng/mL) alone demonstrated approximately a one-minute lag period prior to detection of O_2^- production. When cells were simultaneously exposed to PAF (10^{-6} mol/L) and PMA, O_2^- production was detected almost immediately and the maximal rate (V_{max}) of O_2^- generation was enhanced. When PAF was added 15 minutes prior to PMA, no shortening of the lag period was seen, although the V_{max} was still enhanced (data not shown). When PAF was added after PMA, an immediate increase in V_{max} was demonstrable (V_{max} = nmols O_2^- min). Curves are representative of those obtained using cells from several different donors.

Effect of structurally similar lipids on oxidative response. To determine the specificity of PAF in enhancing O_2^- production, we studied the ability of several structurally-related lipids to enhance responses to FMLP. Because PAF consists of a glycerol backbone with an ether-linked C_{16} or C_{18} saturated fatty acid at position 1, an acetyl group at position 2, and a phosphorylcholine at position 3, we chose compounds with side chains similar to PAF in at least one of three positions. Figure 4 demonstrates dose-response curves for representative lipids. Removal of the acetyl group from position 2 of PAF yielded a compound (lyso-PAF) with 52% of the enhancing activity of PAF at 10^{-5} mol/L. Lyso-PC (substitution of a palmitoyl group for the ether-linked moiety of lyso-PAF) and monopalmitin (removal of the phosphorylcholine group from lyso-PC) had enhancing potency roughly equal to that of lyso-PAF at 10^{-5} mol/L (data not shown). Diolein, a diacylglycerol, had no significant enhancing capabilities. Neither did glycerophosphorylcholine have any effect (data not shown). In contrast, another diacylglycerol, 1-oleoyl, 2-acetyl glycerol, had enhancing activity of magnitude similar to PAF at 10^{-5} mol/L, although PAF was a more potent enhancer at lower concentrations. These results

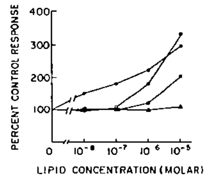

Fig 4. Enhancing effects of structurally similar phospholipids. Neutrophils (1.5 × 10^6 cells) were exposed to the indicated concentration of lipid immediately prior to addition of 10^{-7} mol/L of formyl-methionyl-leucyl-phenylalanine (FMLP) and incubation for five minutes at 37 °C. Results are expressed as percentage of O_2^- production in control cells incubated with FMLP alone. Data points represent means of experiments utilizing neutrophils from at least two different donors. PAF (●—●); Lyso-PAF (■—■); diolein (▲—▲); 1-oleoyl, 2-acetyl glycerol (○—○).

suggest that the fatty acid moiety and diacylglycerol-like activity are important components of the enhancing properties of PAF.

DISCUSSION

PAF triggers neutrophil aggregation, degranulation, and chemotaxis, and is a weak stimulus of O_2^- production. In addition to these direct effects, PAF acts as a regulator of neutrophil responses to other stimuli. Lin et al[12] have shown that PAF desensitizes neutrophils to aggregation induced by LTB_4. Jouvin-Marche et al demonstrated that PAF enhanced the O_2^- response of neutrophils to zymosan particles.[2] The degree of enhancement was consistent with an additive effect of the two oxidative stimuli, in agreement with our own studies using the same particulate stimulus. Ingraham et al[20] demonstrated that PAF enhanced oxygen uptake in neutrophils exposed to FMLP. The present studies suggest that PAF modulates neutrophil O_2^- production in response to the chemotactic peptide FMLP and the phorbol ester PMA and synergistically enhances the oxidative response to these soluble stimuli. Enhancement could be demonstrated without a preincubation period and without the presence of extracellular calcium or magnesium. It was not reversed by removal of PAF from the medium prior to FMLP or PMA exposure.

The mechanism through which PAF accomplishes enhancement remains unclear, although several potential hypotheses seem unlikely in light of our data. Because PAF stimulates LTB_4 production in neutrophils, some investigators have suggested that cellular responses to the phospholipid may be mediated by LTB_4.[12] Our demonstration that both LTB_4 and PAF enhance the oxidative response to FMLP is in keeping with this hypothesis.[10] Although an attractive explanation, it seems unlikely for several reasons. First, enhancement of the response to FMLP by LTB_4 is seen only after cells are exposed to the leukotriene for several minutes prior to addition of the peptide.[10] In contrast, no preincubation period is necessary using PAF as the enhancing agent. Second, we noted marked augmentation of the response to PMA in PAF-treated cells, whereas LTB_4 had no effect on the response of cells to the phorbol ester.[10] Third, the lipoxygenase inhibitor ETYA did not eliminate PAF-mediated enhancement. Thus, it seems likely that PAF exerts its enhancing effect on neutrophil O_2^- production by a mechanism independent of the generation of LTB_4. Alternatively, it is possible that enhancement is mediated by an increase in cellular uptake of calcium. The finding that enhancement of FMLP-induced and PMA-induced oxidative responses by PAF occurred even in the absence of extracellular Ca^{2+} suggests that the mechanism of PAF enhancement does not involve transport of Ca^{2+} into the cell. From our data, however, we cannot rule out an effect on intracellular Ca^{2+} metabolism which could result in a heightened response to soluble stimuli.

It has been shown that preincubation of neutrophils with any of a number of oxidative stimuli results in potentiation of subsequent responses to heterologous oxidative stimuli.[8,10,21] McPhail et al[21] studied this phenomenon and described the cellular response to the first stimulus as "priming." These authors suggested that although oxidative stimuli differ in the transductional mechanisms, they share a common intermediate pathway. One attractive intermediate is protein kinase C, which is activated during neutrophil stimulation by several oxidative stimuli.[22-24] Such stimuli may act through protein kinase C-mediated phosphorylation of neutrophil proteins involved in the generation of O_2^-. A priming stimulus may activate protein kinase C in such a way that response to a second stimulus also working through the kinase is augmented. PAF may be another primary oxidative stimulus acting through this intermediate to enhance responses to other stimuli. In our studies, 1-oleoyl, 2-acetyl glycerol, a diacylglycerol known to stimulate protein kinase C activity, also enhanced O_2^- production, in agreement with a recent report by DeWald et al.[25] Therefore, it is also possible that the enhancing effect of PAF is also mediated through the kinase. Our failure to demonstrate enhancement with diolein, a long-chain diacylglycerol, is consistent with reports showing that this compound does not activate protein kinase C in intact cells, presumably because of the lipid's inability to intercalate into the plasma membrane.[26]

The priming effect of PAF appears to be virtually instantaneous, as shown by continuous assays of O_2^- generation. Such rapid responses may be mediated through direct effects on the neutrophil plasma membrane. The fact that many lipids, including lyso-PC (structurally similar to PAF) are detergents suggests that PAF may also have detergent-like properties. Such properties may alter membrane configuration in such a way that the activity of the O_2^--forming NADPH oxidase system is augmented.

PAF is produced by activated neutrophils and thus may be present in extracellular fluid in inflammatory foci. The lipid may serve to increase oxygen metabolite release at these sites, potentially enhancing both the microbicidal and tissue-damaging properties of neutrophils. The modulation of neutrophil O_2^- generation by PAF is just one of many described instances of regulation of the oxidative response. These regulatory points, whether they be enhancing or inhibitory, provide multiple checks and balances on the inflammatory response. Such mechanisms ensure that this potent immunologic weapon can be held in check until the time when the rapid, powerful neutrophil metabolic burst offers maximal benefit to the host.

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B Cell Growth Factor-Induced Proliferation of Hairy Cell Lymphocytes and Inhibition by Type I Interferon In Vitro

By Kathryn A. Paganelli, Sharon S. Evans, Tin Han, and Howard Ozer

Malignant B cells from hairy cell leukemia (HCL) patients are unable to proliferate when stimulated with standard B cell mitogens. Using chromatographically purified B cell growth factor (BCGF), HCL can be stimulated to proliferate as assessed by incorporation of tritiated thymidine (^{3}H-TdR) into DNA. Proliferation was found to be time dependent, with no detectable ^{3}H-TdR incorporation in up to three days of culture, and significant stimulation evident at days 6 and 10. The presence of 10% BCGF in culture was an absolute requirement for HCL proliferation; however, this BCGF-induced DNA synthesis could be further augmented by the addition of anti-immunoglobulin heavy chain antibodies. BCGF-induced proliferation was abrogated in six of six patients by addition of 1,000 U/mL of recombinant α_2-interferon (IFN) at day 0, although 1,000 U/mL of recombinant γ-IFN had no inhibitory effect in five of six patients studied. Specific cellular receptors for type I IFN were demonstrated in HCL by inhibition of binding of ^{125}I-α_2-IFN by a 40-fold excess of unlabeled α_2 or β IFN with no inhibition by unlabeled γ-IFN. These data demonstrate that malignant HCL lymphoblasts express specific type I IFN receptors and that type I, but not type II IFN, can inhibit growth factor-induced DNA synthesis by hairy cells in vitro. They further suggest a direct antiproliferative mechanism of action for IFN in HCL and predict equivalent clinical activity by either α or β, but not γ IFN in this malignancy.

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ALTHOUGH there has been a great deal of controversy over the origin of the malignant cell in hairy cell leukemia (HCL), recent evidence presented by Korsmeyer et al demonstrates unequivocally that hairy cells have immunoglobulin gene rearrangements characteristic of B cells,[1] providing a convincing argument for the B cell origin of the HCL cell at the genetic level. Among the restrictions imposed in elucidating the biology of this leukemia is the fact that hairy cells are relatively unresponsive to induction of proliferation by standard B cell mitogens.[2,3] Reports on the ability of mitogen-stimulated T cell supernatants and purified B cell growth factor (BCGF) to stimulate normal[4-8] and leukemic[9] human B cells to proliferate in short-term and long-term culture led us to test BCGF on malignant HCL B cells to determine whether this growth factor would support their growth and proliferation. We report here data demonstrating that chromatographically purified BCGF induces proliferation of hairy cells in short-term culture as determined by tritiated thymidine incorporation, and that anti-immunoglobulin heavy-chain antibodies can further augment this response.

The low proliferative index of HCL cells with standard B cell mitogens has previously hindered in vitro studies on hairy cells, especially in terms of cytogenetic analysis and determination of antiproliferative drug effects. In response to recent reports of dramatic clinical remissions induced by α-interferon (IFN) therapy in HCL,[10,11] we used BCGF-induced HCL proliferation to examine first the direct effects of types I and II recombinant IFN on the proliferative response of HCL cells to BCGF, and second the expression and specificity of IFN receptors on HCL cells. Although previous studies of IFN receptor expression have been performed almost exclusively on lymphoblastoid[12-15] and fibroblast[16] cell lines, the data presented here support the contention that HCL cells express specific type I receptors and that binding of recombinant α-IFN to these receptors can directly inhibit growth factor-induced DNA synthesis in this malignancy.

MATERIALS AND METHODS

Reagents. BCGF was obtained from Cellular Products, Inc, Buffalo. This factor has been chromatographically purified from crude mitogen-stimulated peripheral lymphocyte supernatants and is devoid of interleukin 2 (IL-2) activity but capable of sustaining the growth of normal B lymphocytes. Polyclonal rabbit anti-human μ and γ chain antibodies coupled to polyacrylamide beads were purchased from Bio-Rad Laboratories, Rockville Centre, NY. Recombinant α_2-IFN was obtained from Schering Plough Corporation, Kenilworth, NJ; β-IFN was obtained from Cetus Corporation, Emeryville, Calif; and γ-IFN was obtained from Biogen Inc, Cambridge, Mass. All interferons (α_2, β, and γ) were recombinant bacterial products purified to > 99% homogeneity

Preparation of affinity-purified rabbit anti-human IgM (soluble anti-μ). Pure IgM was obtained by passage of the ammonium sulfate-precipitated immunoglobulin fraction of an IgM macroglobulinemia patient's serum over a Sephacryl-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column. Recovered IgM was determined to be free of IgG and IgA by ELISA and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This IgM was used to immunize a rabbit to human IgM and to couple to an affinity column for recovery of anti-IgM. The affinity column was prepared by coupling IgM to cyanogen-bromide–activated Sepharose-4B (Pharmacia) according to manufacturer's directions. The IgM-coupled gel was packed into a 3-mL syringe, and serum from the IgM-immunized rabbit was recirculated through the gel overnight at room temperature by means of a peristaltic pump The syringe was washed with at least 10 bed vol of phosphate-buffered saline (PBS), and the coupled anti-human IgM was eluted with 0.1 mol/L of glycine HCl containing 1 mol/L of NaCl (pH 2.5). The eluted fractions were pooled and dialyzed against PBS. Any reactivity to IgG was removed by absorbing the anti-IgM eluate with IgG coupled to Sepharose-4B.

Cell separation. Eight patients with HCL in leukemic phase were used as peripheral blood donors for these studies. All were men,

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ranging in age from 37 to 75 years. Mononuclear cells were separated from heparinized blood samples by Ficoll-Hypaque density gradients. Recovered cells were washed and resuspended in RPMI 1640 medium supplemented with antibiotics, 25 mmol/L of HEPES buffer and 20% fetal calf serum (FCS) This cell population was T cell depleted by rosetting overnight at 4 °C with AET-modified sheep red blood cells (SRBCs).[17,18] Nonrosetting cells (hereinafter referred to as B cells) were removed from the interface of a second Ficoll gradient. The percentage of hairy cells in this population was routinely assessed by morphological examination of cytocentrifuge preparations stained with Wright's-Giemsa. Staining for tartrate-resistant acid phosphatase (TRAP) was also performed on duplicate slides.[19] Latex bead ingestion[20] was determined by incubating 1×10^6 cells with 10 μL of a 1:10 dilution of 1.1-μm latex particles (Sigma Chemical Co, St Louis, LB-11) in a final 1 mL vol. After a one-hour incubation at 37 °C in a humidified atmosphere of 5% CO_2 in ambient air, cells were washed and cytocentrifuge preparations were made. Slides were stained with Wright's-Giemsa, and cells ingesting two or more latex particles were assessed as positive for phagocytic activity.

Normal human B cells were isolated from buffy coats as follows. peripheral blood buffy coat concentrate was withdrawn from a sterile transfer pack into a heparinized syringe and centrifuged for 30 minutes at 1,500 rpm over a Ficoll gradient. Mononuclear cells from the interface were depleted of adherent cells by incubating them for one hour at 37 °C in a 30-mL vol of RPMI 1640/10% FCS in a 75-cm^2 plastic flask (Corning 25110). The nonadherent cells were poured off, washed, and subsequently depleted of T cells by AET-SRBC rosetting for 90 minutes on ice. After Ficoll separation, the T-depleted lymphocyte population was put through a second one-hour adherence step at 37 °C and a second rosetting step at 4 °C for 18 hours. After removal of rosetted T cells by Ficoll, the resulting population was used as the source of normal B cells

Proliferation assay for HCL B cells and normal B cells. Cultures were performed in 96-well flat-bottomed microtiter plates (Corning 25860) with 1×10^5 cells per well in a 0.2-mL vol. Factors were added to cultures as described in legends to Figs 1 through 5. Incubations for indicated time periods were carried out at 37 °C in a humidified 5% CO_2–air atmosphere; 2 μCi per well of methyl-^{3}H-thymidine (6.7 Ci/mmol) (New England Nuclear, Boston) were added, and harvesting of cells onto glass microfiber filters was performed 24 hours later. Filters were placed in scintillation vials containing 7 mL of a mixture of 170 mL of Spectrafluor PPO-POPOP (Amersham Corporation, Arlington Heights, Ill) to 4 L of toluene (J.T. Baker Chemical Co, Phillipsburg, NJ) and counted in a beta scintillation counter (Tri-Carb 460 C, Packard Instrument Co, Inc, Downers Grove, Ill).

TCGF assay. BCGF was confirmed to be free of IL-2 activity by both a thymidine incorporation assay and a five-day growth assay, comparing BCGF with a TCGF standard for activity on activated T cells.

Target T cells were prepared by a seven-day activation of human peripheral lymphocytes prepared from Ficoll-separated buffy coats. T cells were stimulated with 0.5% vol/vol phytohemagglutinin-p (PHA-P) (Difco Laboratories, Detroit) in RPMI 1640 containing 15% FCS and 50 μg/mL of gentamycin at a cell density of 1×10^6/mL. Cultures were held at 37 °C for seven days, at which time activated T cells were recovered and washed three times in RPMI 1640.

Thymidine incorporation was assessed by placing 1×10^5 activated cells per well in a 96-well plate along with twofold dilutions of either BCGF or a TCGF standard, beginning at 25% concentration of factor. Cells were diluted in RPMI 1640 containing 15% FCS, 50 μg/mL of gentamycin, and 2 mmol/L of glutamine. After 48 hours of incubation at 37 °C, cultures were pulsed with 0.25 μCi of tritiated thymidine per well and were harvested after 24 hours

Five-day growth assays were carried out, using the same population of activated T cells: 2×10^5/mL T blasts were placed in 1-mL aliquots in 24-well trays with final concentrations of 0% to 20% BCGF or TCGF standard. Cultures were counted after five days at 37 °C and compared with the TCGF standard.

Iodination of α_2-IFN. Iodination was carried out by the iodogen method, essentially as described by Markwell and Fox.[12] In brief, 10 μg of α_2-IFN (5×10^4 U) were combined with 0.5 mCi of Na ^{125}I in a 30-μl vol and allowed to react for 30 minutes at room temperature. Free ^{125}I was separated from bound ^{125}I by fractionation on a Sephadex G-25 column. The radioactive peaks were pooled and tested for antiviral activity by challenge of GM2504 cells with vesicular stomatitis virus, with assessment of protection from viral cytopathic effect according to Finter's method. α_2-IFN was iodinated to a final specific activity of 32 mCi/mg of protein and retained >90% of its antiviral activity.

Cellular binding of ^{125}I-α_2-IFN. Specific binding of ^{125}I-α_2-IFN to hairy cells was performed by modification of the method of Branca and co-workers.[14,15] In brief, 5×10^6 or 1×10^7 cells were incubated in plastic 12 × 75-mm tubes (Falcon, Oxnard, Calif) with 0 5 to 50 ng of ^{125}I-α_2-IFN in a final 1-mL vol of RPMI 1640/20% FCS for one hour in a 37 °C water bath. Duplicate tubes containing a 40-fold excess of unlabeled α_2-IFN in addition to the labeled IFN were included to quantitate nonspecific binding. Unbound ^{125}I-α_2-IFN was removed by three washes with 0.1% BSA/PBS, and the dry cell pellets were counted in a Packard model 5260 gamma counter. Specificty of binding was determined by the formula. percentage of specific binding = uninhibited CPM − inhibited CPM/inhibited CPM × 100.

RESULTS

Cell populations. HCL B cells (E rosette negative) in all cases were > 90% typical HCL cells by morphological criteria. TRAP positive cells in these populations ranged from 4% to 82%, and latex phagocytizing cells ranged from 33% to 92%.

Thymidine incorporation of peripheral blood HCL B cells. As seen in Fig 1, BCGF stimulated thymidine uptake of HCL cells in a time-dependent manner. No thymidine was

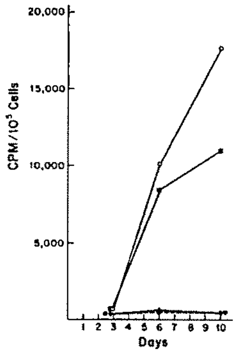

Fig 1. ^{3}H-Thymidine incorporation by hairy cell leukemia (HCL)-B cells. E rosette-negative cells were incubated for 3-, 6-, and 10-day periods with medium alone (RPMI 1640/20% fetal calf serum), 10% B cell growth factor (BCGF), 15 μg/mL soluble rabbit anti-human IgM, or BCGF + anti-IgM. Triplicate cultures of 1×10^5 cells per well were incubated in a final 0.2-mL-vol. ■, BCGF; ○, BCGF + anti-μ; ♦, anti-μ; ●, control.

incorporated in up to three days of culture. At day 6, proliferation was readily detected in BCGF-stimulated cultures, with ^{3}H-TdR incorporation increasing from day 6 to day 10, the last measurement. A combination of BCGF and anti-μ or anti-γ stimulation was also effective in initiating DNA synthesis, with increased activity of the combined stimuli on day 10. No proliferation was observed unless BCGF was present. The effect of anti–heavy-chain antibodies on different patients varied. Although no patient's cells showed spontaneous proliferation in the absence of BCGF and seven of eight responded to addition of BCGF with proliferation of > 1,000 cpm/10^5 cells, most patient samples demonstrated a significantly augmented response when anti–heavy-chain antibodies were added to cultures along with BCGF, and some showed similar levels of BCGF-stimulated DNA synthesis with or without anti–heavy-chain antibodies (Fig 2). In one patient who responded to BCGF with < 1,000 cpm (patient 4, Table 1), significant proliferation was achieved when insoluble anti-μ was added. In addition, in two patients for whom soluble anti-μ was compared to insoluble anti-μ, the insoluble form gave greater stimulation of DNA synthesis than did the soluble form. Overall, of seven patients tested with insoluble anti–heavy-chain antibodies, proliferation in five of the seven was significantly greater in the presence of the anti–heavy-chain antibodies. The remaining two patients showed only marginal augmentation in the presence of anti–heavy-chain antibodies.

Figure 3 shows the differential effect of α_2-IFN and γ-IFN on thymidine uptake. Neither recombinant α_2-IFN nor γ-IFN directly induced proliferative activity by HCL cells. However, the results indicate that 1,000 U/mL of α_2-IFN was capable of inhibiting BCGF-induced proliferation by cultured malignant HCL B cells. In contrast, 1,000 U/mL of recombinant γ-IFN did not have an antiproliferative effect when added to HCL cells in the presence of BCGF. As seen in Table 1, six of six patients tested for inhibition of proliferation by different IFN species were severely reduced in their ability to synthesize DNA in response to BCGF when cultured in the presence of 1,000 U/mL of α_2-IFN. Thymidine incorporation in the presence of both BCGF and α_2-IFN ranged between 6% and 35% of values obtained with BCGF alone, with a mean value of 17.5%. By the Wilcoxon test, the reduction in thymidine incorporation was significant with $P = .028$ when comparing BCGF alone with BCGF plus α_2-IFN. In contrast, γ-IFN was substantially inhibitory in only one case, patient 4. In the remaining five patients, γ-IFN enhanced proliferation in four and minimally reduced proliferation in one. The percentage of BCGF control ranged from a low of 21% in patient 4 to 70% to 190% in the remaining patients, with a

Table 1. DNA Synthetic Response of Leukemic Cell Populations From Eight HCL Patients to BCGF/IFN

Patient No	CPM Control	CPM BCGF Alone	BCGF α_2-IFN (% of BCGF Control)	BCGF γ-IFN (% of BCGF Control)
1	147	6,982	429 (6)	13,253 (190)
2	346	4,971	1,744 (35)	5,824 (117)
3	144	1,213	202 (17)	1,533 (126)
4*	125	506	1,725	969
		16,975*	1,581* (9*)	3,622* (21*)
5	208	2,202	283 (13)	1,534 (70)
6	577	17,676	4,482 (25)	28,235 (160)
7	41	2,442	NT —	NT —
8	141	4,137	NT —	NT —
			Mean	Mean
			17.5%	114%
			$P = .028$	$P = 0 60$

HCL, hairy cell leukemia; BCGF, B cell growth factor; IFN, interferon

For seven days, 1 × 10^5 cells per well were incubated with medium, 10% BCGF, 1,000 U/mL α_2-IFN or γ-IFN, and combinations of the above

*Because this patient did not proliferate to BCGF alone, the starred values give cpm when anti-μ was included with BCGF, and percentage of BCGF control is based on these values

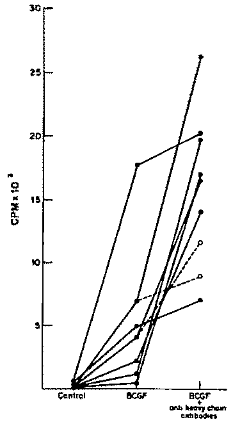

Fig 2. Effect of anti-μ or anti-γ on B cell growth factor (BCGF)-induced hairy cell proliferation. Cells from seven different hairy cell leukemia (HCL) patients were incubated in medium alone, with 10% BCGF, or 10% BCGF + 15 μg/mL soluble or insoluble anti-immunoglobulin heavy-chain antibodies for seven days. Thymidine was added for an additional 24 hours prior to harvesting. ●–●, insoluble; ○——○, soluble.

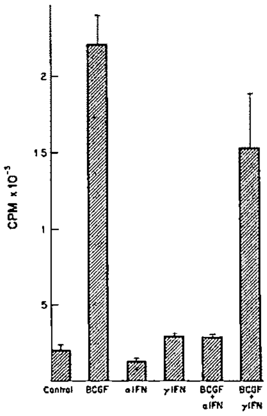

Fig 3. Effect of α_2-interferon (IFN) and γ-IFN on ^{3}H-thymidine incorporation. 1 × 10^5 hairy cell leukemia (HCL)-B cells per well (in triplicate) were incubated for seven days with medium alone, 10% B cell growth factor (BCGF), 1,000 U/mL of recombinant α_2-IFN, 1,000 U/mL of recombinant γ-IFN, or BCGF in combination with α-IFN or γ-IFN (cpm values represent the mean ± SE).

mean value of 114% ($P = .60$). It is clear that α_2-IFN and γ-IFN have differential effects on BCGF-induced proliferation of malignant hairy cells.

Effect of IFN on normal peripheral blood B cell proliferation. The normal B cell response to BCGF and anti-μ was qualitatively similar to the proliferation seen with hairy cells (Table 2). As with hairy cells, addition of 1,000 U/mL of recombinant α_2-IFN had an antiproliferative effect on BCGF/anti-μ–stimulated B cells. In addition, normal B cell proliferation was inhibited to some extent by γ-IFN, although α_2-IFN reduced proliferation to a greater degree than did γ-IFN (65% inhibition with α_2-IFN as compared with 20% to 39% inhibition by γ-IFN).

Lack of IL-2 activity in BCGF preparations. BCGF, supplied by Cellular Products, was confirmed to be free of IL-2 activity by both a ^{3}H-thymidine incorporation assay (Fig 4) and by a five-day growth assay (Table 3). A TCGF standard stimulated proliferation of T cell blasts in both thymidine incorporation and growth assays, whereas BCGF had no stimulatory activity in either assay.

Specific binding of ^{125}I-α_2-IFN by HCL cells. The binding curve shown in Fig 3 was generated by incubating HCL cells with ^{125}I-α_2-IFN in concentrations ranging from 0.5 to 50.0 ng per 10^7 cells, with or without a 40-fold excess of unlabeled α_2-IFN. Binding of ^{125}I-α_2-IFN increased up to the highest concentration tested and was inhibited by unlabeled recombinant α_2-IFN with a specificity of 69% to 86%. Saturation of binding sites was approached with increasing doses of ^{125}I-α_2-IFN (Fig 5). Scatchard analysis[21] (Fig 5, inset) revealed the apparent dissociation constant (Kd) to be 7.1×10^{-10} mol/L, indicating a high-affinity binding site. The approximate number of IFN receptors per cell (also based on Scatchard analysis) was calculated to be ~350.

A competitive inhibition experiment demonstrated that unlabeled recombinant α_2-IFN or β-IFN but not γ-IFN can effectively compete with ^{125}I-α_2-IFN for binding sites on HCL cells. α_2-IFN inhibited binding by 75.4%, β-IFN inhibited it by 67.9%, and γ-IFN showed no inhibition. This strongly supports the concept that α_2-IFN and β-IFN share a common receptor on malignant HCL cells and that the γ-IFN receptor, if present, is distinct, in agreement with the findings of Branca and Baglioni of unique receptors for types I and II IFNs on lymphoblastoid cell lines.[14]

Table 2. Seven-Day Thymidine Incorporation of Normal B Cells

Factor Added	Exp 1 (cpm)	Exp 2 (cpm)
Control	1,223	6,605
BCGF	14,161	16,955
Anti-μ	283	1,275
BCGF + anti-μ	11,543	13,079
BCGF, anti-μ, α-IFN	4,059	4,648
BCGF, anti-μ, γ-IFN	7,016	10,496
α-IFN	256	2,781
γ-IFN	259	2,997

Normal peripheral blood B cells were cultured for seven days at 1×10^6 cells per well (in triplicate) with RPMI 1640 and 20% FCS. Additions were as indicated: BCGF 10%, anti-μ 15 μg/mL (Bio-Rad 170-5120), α_2-IFN 1,000 U/mL, and γ-IFN 1,000 U/mL

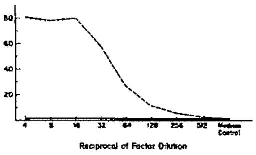

Fig 4. B cell growth factor (BCGF) was compared to a TCGF standard in assessing the presence of interleukin 2 in factor preparations. Phytohemagglutinin-activated T cells were added to dilutions of BCGF or TCGF at 1×10^5 per well, cultured for 48 hours at 37 °C, and then pulsed for 24 hours with ^{3}H-thymidine and harvested. ———, TCGF; ——, BCGF.

DISCUSSION

In our experience, BCGF has proven to be useful in stimulating HCL cells to divide in vitro. This is an important finding because standard B cell mitogens are relatively ineffective in inducing HCL proliferation. To date, no consistent or diagnostic chromosomal abnormality has been found in HCL, possibly due to the difficulty encountered in obtaining analyzable metaphases. BCGF may prove useful in extending cytogenetic or molecular genetic studies of HCL by stimulating cell division to a more easily quantitated level.

The effect of anti-heavy chain antibodies on BCGF-induced hairy cell proliferation was variable but, in most patients, it augmented BCGF-associated proliferation. However, anti–heavy-chain antibodies were not an absolute requirement for proliferation, as BCGF alone gave significant amounts of thymidine incorporation in the absence of anti-μ or anti-γ in most patients. Two subsets of B cells have been described by Muraguchi et al[7]: (a) a small, resting population of B cells that requires an activation step prior to becoming BCGF responsive; and (b) a larger, activated population that can respond directly to BCGF in the absence of prestimulation. This second subset may have been activated in vivo. It seems likely that some proportion of hairy cells fall into the second category, as they are large B cells that are capable of direct response to BCGF in the absence of activation by anti–heavy-chain antibodies.

BCGF may also prove useful in in vitro drug sensitivity testing on hairy cells and possibly other B-derived lympho-

Table 3. Lack of IL-2 Activity in BCGF Preparation

Factor Concentration (%)	Five-Day Cell Count ($\times 10^5$/mL)	
	TCGF	BCGF
20.00	22.6	0.4
10.00	22.7	0.7
5.00	18.3	0.2
2.50	6.1	0.4
1.25	4.2	0.4
0	0.5	0.5

Seven-day T cell blasts were prepared as described and added to 24-well plates at a starting concentration of 2×10^5/mL with varying concentrations of a T cell growth factor (TCGF) standard or with B cell growth factor (BCGF) to determine that no interleukin 2 activity was present in BCGF Plates were incubated for five days, and cell counts were determined by trypan blue exclusion.

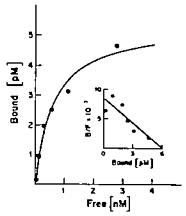

Fig 5. Specific binding of $^{125}I-\alpha_2$ interferon (IFN) to hairy cell leukemia cells. Lymphocytes (10^7) were incubated at 37 °C for one hour with 0.5 to 50 ng $^{125}I-\alpha_2$-IFN. Values shown are corrected for nonspecific binding, as assessed by inclusion of a 40-fold excess of unlabeled α_2-IFN. The Scatchard plot (inset) was generated by linear regression.

proliferative malignancies. To test antiproliferative capacity of a cell cycle-specific drug on tumor cells, measurable proliferation must be obtained prior to drug addition. In our studies with BCGF in combination with α_2-IFN and γ-IFN, α_2-IFN appears to inhibit DNA synthesis by the dividing hairy cells directly, whereas γ-IFN had no inhibitory effect in most patients tested and may even increase proliferation in some patients. The differential effects of types I (α and β) and II (γ) IFNs may be related to the fact that type I and II receptors are distinct, each IFN binding specifically to its own receptor.[14] It is likely that the biological effects and possible that the therapeutic effects of type I and II IFNs are also distinct. These observations may prove useful in determining the efficacy of different types of IFNs for therapeutic use, and the in vitro data predict that γ-IFN may have limited efficacy in this malignancy.

It is interesting to speculate on the mechanism of action of α_2-IFN in its successful treatment of hairy cell leukemia as compared with the less dramatic responses seen with other B cell tumors.[22] We have found α_2-IFN receptors on other types of malignant B cells from patients whose disease is relatively unresponsive to IFN therapy (data not shown), indicating that simple presence of receptors does not confer sensitivity to the therapeutic effects of IFN. As suggested by Hannigan et al,[13] resistance may be due to the lack of growth inhibitory effects of IFN on IFN-resistant cells, rather than to the absolute presence or absence of receptors. The fact that α_2-IFN directly inhibits hairy cell proliferation and γ-IFN does not suggests the possibility that α_2-IFN is exerting its therapeutic effects at least partially by direct inhibition of malignant cell growth rather than indirectly as a biological response modifier of non malignant cells.

Unlabeled α_2-IFN and β-IFN were each able to inhibit binding of $^{125}I-\alpha_2$-IFN to hairy cells, whereas γ-IFN was not capable of competing for the α_2-IFN binding site, indicating the presence of unique receptors for type I interferon on malignant B cells, as found on B cell lines.[14] These data indicate that IFN's therapeutic effects may be mediated by receptor-dependent phenomena in HCL. It may be possible to gain a clearer understanding of this interaction and its significance by attempting to modulate receptor expression in vitro by treatment with IFN and other lymphokines or by monitoring receptor expression in patients before and during IFN therapy.

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Impaired Expression of Cell Surface Receptors for B Cell Growth Factor by Chronic Lymphocytic Leukemia B Cells

By Robert T. Perri

Normal human B cell proliferation is controlled by various immunoregulatory signals including the T cell-derived lymphokine B cell growth factor (BCGF). The role of BCGF in the regulation of malignant B cell proliferation is unclear. Therefore, we studied the proliferative response of purified chronic lymphocytic leukemia (CLL) B cells to BCGF. For all CLL patients studied, CLL B cells showed a decreased proliferative response as compared with control B cells for BCGF-induced B cell proliferation (patient 291 ± 59 cpm *v* control 3,942 ± 622, mean ± SEM). This impaired proliferative response appeared to be intrinsic to CLL B cells since it was not corrected by incubation with increasing concentrations of BCGF. Attainment of normal B cell responsiveness to BCGF requires the processing of an initial activation signal which results in the expression of cell surface receptors for BCGF. Increasing concentrations of the B cell activation signal (the $F(ab')_2$ fragment of goat anti-human μ chain) did not improve CLL B cell responsiveness to BCGF. Three-day activated CLL B cells compared with activated control B cells demonstrated a marked impairment in their ability to absorb out the BCGF activity present in the BCGF preparation (BCGF activity absorbed out, patient 12.8% *v* control 53%). Pretreatment of CLL B cells with neuraminidase failed to improve either the proliferative response to BCGF or the expression of cell surface receptors for BCGF by the CLL B cells. This study suggests that the impaired responsiveness to BCGF by CLL B cells is the result of impaired expression of cell surface receptors for BCGF when CLL B cells are exposed to activation signals.

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MOST CASES of chronic lymphocytic leukemia (CLL) represent a monoclonal expansion of B cells.[1] Most CLL B cells are not actively dividing and appear to be resting in the Go phase of the cell cycle.[2] Previous studies have demonstrated that CLL lymphocytes have an impaired response to the mitogenic signals of the plant lectins concanavalin A (Con A), phytohemagglutinin (PHA), and pokeweed mitogen (PWM).[3–5]

Normal human B cell proliferation is controlled by various immunoregulatory signals, including the T cell-derived lymphokine B cell growth factor (BCGF)[6–8] The stimulation of resting B cells to proliferate requires two signals.[9–12] The first signal required by the resting B cell is considered an activation signal. This activation signal results in the expression of cell surface receptors for BCGF. The expression of BCGF receptors provides the B cell with the capacity to respond to the proliferation signal delivered by BCGF. Normal human B cells are driven from their resting state to a proliferative state by this two-signal process.[9–12]

The role of BCGF in the regulation of malignant B cell proliferation is unclear. Previous studies have described autostimulatory activity produced by Epstein-Barr virus (EBV)-positive B lymphoblast cell lines.[13–16] In Namalva, an EBV-positive human B cell lymphoma line, this autostimulatory activity appears to be provided by BCGF.[16] A recent report identified malignant B cells from a patient with poorly differentiated lymphocytic lymphoma that proliferated in response to anti-human IgM alone.[17] Proliferation of this patient's malignant B cells was not enhanced by the addition of BCGF.

In the present study, we examined further the role of BCGF in the regulation of malignant B cell proliferation by studying the responsiveness of CLL B cells to the proliferation signal delivered by BCGF.

MATERIALS AND METHODS

Patients. After informed consent was obtained, B-CLL patients and normal control donors matched as closely as possible for age and sex were studied. When studied, none of the patients were receiving chemotherapy. Some patients had received chemotherapy within the month prior to the study.

Cell separations. Peripheral blood mononuclear cells were isolated from venous blood by Ficoll-Hypaque. Monocyte depletion was done by adherence in tissue culture flasks for 45 minutes at 37 °C in 5% CO_2. Non-adherent cells were harvested and were then allowed to rosette with AET-sensitized sheep erythrocytes (SRBCs) at 4 °C for two hours. Rosetted T cells were separated from the nonrosetted B cells by two successive Ficoll gradients. SRBCs were removed by hypotonic lysis with distilled H_2O. The twice nonrosetted B cell-enriched population (80% to 90% sIg+) was used in the following studies. T cell contamination in this population was <1% as determined by OKT3 reactivity. In addition, no mitogenic effect was noted when the B cell-enriched population was incubated with PHA (Difco Laboratories Inc, Detroit).

B Cell proliferation assay. This assay was performed as previously reported.[18] In brief, B cell proliferation was studied by culturing 5×10^4 control or CLL B cells obtained as described above in 0.2 mL of RPMI 1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS) (Gibco) in 6-mm flat-bottomed microtiter wells (Linbro, Hamden, Conn). All experiments were done in triplicate. Plates were secured tightly and incubated at 37 °C with 5% CO_2 for three days. Various reagents were added to the cultures as indicated. In the standard proliferation assay, cells were cultured in the presence or absence of 10% BCGF (Celluluar Products, Buffalo) together with 1% (vol/vol) staphylococcal Protein A (SPA) (Sigma, St Louis) or goat $F(ab')_2$ anti-human IgM chain (Cappel, Cochranville, Pa) at 15 μg/mL. BCGF was prepared as previously described.[7] The purification scheme yielded a product free of immune interferon, interleukin 1 (IL-1)

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and interleukin 2 (IL-2). This BCGF preparation supports both the short-term proliferation of activated human B cells[7,11,19] and the proliferation of human B cells in long-term culture.[11] This BCGF preparation does not support the proliferation of T cells.[11,19] It is primarily enriched for the human B cell stimulating factor (BSF) that appears to correspond to BSF-pl.[20] Both SPA 1% (vol/vol) and goat $F(ab')_2$ anti-human IgM chain 15 μg/mL gave comparable results when used as the initial activation signals in this B cell proliferation assay. The cultures were pulsed with ^3H-thymidine (1 μCi per well) (6.7 Ci/mmol/L, New England Nuclear, Boston) over the last 16 to 20 hours of incubation, harvested by a MASHII cell harvester in H_2O, and counted in a Packard scintillation spectrometer.

Absorption of BCGF activity by activated B cells Control or CLL B cells were prepared as described above. These control or CLL B cell-enriched populations were cultured for three days in the presence of SPA 1% (vol/vol) or varying concentrations of goat $F(ab')_2$ anti-human IgM chain. After this three-day incubation, 1 × 10^6 mL of viable control or CLL B cells were incubated for two hours at 4 °C in an equal volume mix with the BCGF preparation After this incubation, the preparation was tested for residual BCGF activity in the standard BCGF proliferation assay with freshly isolated control B cells as the target cells. Both SPA 1% (vol/vol) and goat $F(ab')_2$ anti-human IgM chain 15 μg/mL gave comparable results when used as the initial activation signals. Preabsorption values are those seen with the BCGF preparation before exposure to the three-day activated control or CLL B cells. Postabsorption values are those seen after exposure to the activated control or CLL B cells. PHA-stimulated lymphoblasts failed to absorb BCGF activity for the BCGF preparation. Percentage of activity adsorbed is calculated by dividing postabsorption cpm by preabsorption cpm then subtracting this from one, and multiplying that value by 100.

Neuraminidase treatment of B cells. Control or CLL B cells were isolated as described above As previously reported, 1 × 10^6 control or CLL B cells were incubated with neuraminidase (*Clostridium perfringens* 50 U/mL, Sigma) for 30 minutes at 37 °C with constant shaking.[21] The cells were then washed five to seven times and resuspended in RPMI 1640 with 10% FCS prior to their use in the proliferation assays described above.

RESULTS

The responsiveness of CLL B cells to the proliferation signal of BCGF was studied. The proliferative response of CLL B cells to BCGF was markedly impaired (Fig 1). In the presence of both an activation signal and BCGF, control B cells had a stimulation index of 16.7. In contrast, under similar culture conditions, CLL B cells had a stimulation index of only 2.3. All of the CLL B cells we have studied have demonstrated an impaired proliferative response to BCGF.

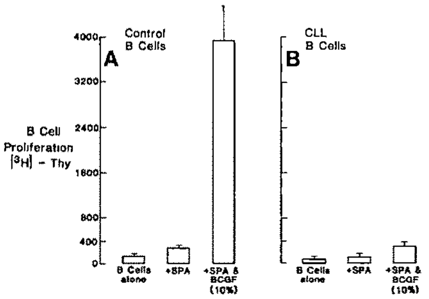

Fig 1. Comparison of (A) control and (B) CLL B cell proliferative responses to BCGF. Cultures were prepared as described in the Materials and Methods section. Data represent the mean ± SEM.

We next attempted to determine if CLL B cells were capable of responding to increased concentrations of BCGF (Fig 2). Control B cells responded to the proliferation signal of BCGF in a dose-dependent manner. In contrast, at all concentrations of BCGF studied, CLL B cells consistently exhibited a marked impairment in their proliferative response to BCGF.

We next sought to determine whether the impaired proliferative response of CLL B cells to BCGF was the result of impaired processing of the initial activation signal. Previous studies have demonstrated that the nature of the B cell activation signal provivded by anti-μ is dependent upon the concentration of anti-μ chosen.[11,12] Initially, we studied the effect of increasing concentrations of anti-μ on the responsiveness of CLL B cells to the proliferation signal of BCGF (Fig 3). As shown, increased concentrations of anti-μ as the initial activation signal did not improve the responsiveness of CLL B cells to the proliferation signal of BCGF.

Impaired processing of an initial activation signal by CLL B cells would be expected to result in decreased or abnormal expression of cell surface receptors for BCGF by the CLL B cells. Because these studies suggested that CLL B cells have an impaired activation response, we sought to determine if this impaired activation response by CLL B cells results in a defective expression of cell surface receptors for BCGF. In previous studies, the presence of receptors for BCGF on activated control B cells has been demonstrated by their ability to absorb out selectively the BCGF activity present in a BCGF preparation.[11] The ability of activated control and CLL B cells to absorb out the BCGF activity present in the BCGF preparation was studied (Table 1). As shown, activated control B cells were capable of absorbing out significant BCGF activity from the BCGF preparation. In contrast, activated CLL B cells demonstrated a marked impairment in their ability to absorb out BCGF activity from the BCGF preparation. These results suggest that CLL B cells have an impaired expression of cell surface receptors for BCGF. This impaired expression of BCGF receptors by CLL B cells may be the result of an impaired processing by CLL B cells of the initial activation signal.

A previous study demonstrated abnormal glycosylation of CLL T cell membrane components.[21] In an effort to determine if the impaired expression of BCGF receptors in response to activation signals by CLL B cells was the result

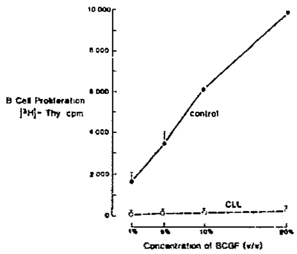

Fig 2. Proliferative response of control and CLL B cells to varying concentrations of BCGF in the presence of anti-μ (15 μg/mL). Cultures were prepared as described in the Materials and Methods section. Data represent the mean ± SEM.

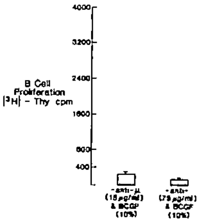

Fig 3. Proliferative response of CLL B cells to BCGF 10% (vol/vol) in the presence of varying concentrations of anti-μ. Cultures were prepared as described in the Materials and Methods section. Data represent the mean ± SEM.

of abnormal glycosylation of CLL B cell membrane components, we examined whether neuraminidase pretreatment of CLL B cells resulted in improved expression by CLL B cells of cell surface receptors for BCGF after an initial activation signal. CLL B cells were pretreated with neuraminidase before incubation with SPA 1% (vol/vol) for three days. The ability of neuraminidase-pretreated, three-day–activated CLL B cells to absorb out BCGF activity from the BCGF preparation was examined (Table 2). Neuraminidase pretreatment of CLL B cells did not result in enhanced expression of CLL B cell surface receptors for BCGF. Both neuraminidase-pretreated and nontreated CLL B cells had a similarly impaired capability to absorb out BCGF activity.

To confirm the failure of neuraminidase pretreatment to enhance CLL B cell BCGF receptor expression, we next studied the effect of neuraminidase pretreatment of CLL B cells on CLL B cell responsiveness to the proliferative signal of BCGF. No improvement in the proliferative response of CLL B cells to BCGF was seen when CLL B cells were pretreated with neuraminidase. Neither increased concentrations of neuraminidase nor longer preincubation times with neuraminidase resulted in any improvement in the proliferative response of CLL B cells to BCGF (data not shown). These results do not support the hypothesis that the impaired proliferative response of CLL B cells to BCGF is the result of abnormal glycosylation of CLL B cell membrane components.

Table 1. Defective Absorption of BCGF by CLL B Cells

BCGF (%) (vol/vol)†	Incorporation of ³H-Thymidine (cpm)* Preabsorption	 Postabsorption by Control B Cells	 Postabsorption by CLL B Cells	 Activity Absorbed (%)‡
10	5,351	2,438	—	54.4
20	7,727	3,633	—	53.0
10	5,351	—	4,495	16.0
20	7,727	—	6,734	12.8

BCGF, B cell growth factor, CLL, chronic lymphocytic leukemia.

*Incorporation of ³H-thymidine refers to the proliferative response of 5 × 10⁴ control B cells to staphylococcal Protein A (SPA) 1% (vol/vol) in the presence of the BCGF preparation, which was added both before and after absorption by SPA-stimulated control and CLL B cells. Cultures were incubated for three days with the addition of ³H-thymidine (1 μCi per well) for the last 16 hours. For the absorption of the BCGF preparation, control and CLL B cells were exposed to SPA 1% (vol/vol) for three days. After this interval, control and CLL B cells (10 × 10⁶ cells) were incubated for two hours at 4 °C with the BCGF preparation. After incubation, the preparation was tested for residual BCGF activity. Preabsorption values are those seen with the BCGF preparation before exposure to the SPA-activated B cells. Postabsorption refers to those values seen after exposure to the activated B cells Activated PHA-stimulated T lymphoblasts failed to absorb the BCGF activity.

†BCGF (vol/vol) refers to the final concentration of the BCGF preparation added to the proliferation assay.

‡Percentage of activity absorbed is calculated by dividing postabsorption cpm by preabsorption cpm, then substracting this from one, and multiplying that value by 100.

Table 2. Effect of Neuraminidase Treatment of CLL B Cells on Absorption of BCGF by CLL B Cells

BCGF (%) (vol/vol)†	Incorporation of ³H-Thymidine (cpm)* Preabsorption	 Postabsorption by CLL B Cells	 Postabsorption by Treated CLL B Cells	 Activity Absorbed (%)‡
10	9,495	7,042	—	25.8
20	13,611	11,110	—	18.4
10	9,495	—	7,469	21.3
20	13,611	—	13,183	3.2

BCGF, B cell growth factor; CLL, chronic lymphocytic leukemia.

*Incorporation of ³H-thymidine refers to the proliferative response of 5 × 10⁴ control B cells to staphylococcal Protein A (SPA) 1% (vol/vol) in the presence of the BCGF preparation, which was added both before and after absorption by SPA-stimulated neuraminidase-pretreated and nontreated CLL B cells. Cultures were incubated for three days with the addition of ³H-thymidine (1 μCi per well) for the last 16 hours. For the absorption of the BCGF preparation, CLL B cells were exposed to SPA 1% (vol/vol) for three days. Neuraminidase-treated and nontreated CLL B cells (10 × 10⁶ viable CLL B cells) were incubated then for two hours at 4 °C with the BCGF preparation. After incubation, the preparation was tested for residual BCGF activity. Preabsorption values are those seen with the BCGF preparation before exposure to the SPA-activated cells Postabsorption refers to those values seen after exposure to the activated cells. Activated PHA-stimulated T lymphoblasts failed to absorb the BCGF activity. Neuraminidase treatment of control B cells did not alter their absorption of BCGF.

†BCGF (vol/vol) refers to the final concentration of the BCGF preparation added to the proliferation assay.

‡Percentage of activity absorbed is calculated by dividing postabsorption cpm by preabsorption cpm, then substracting this from one, and multiplying that value by 100.

DISCUSSION

In this study, we have demonstrated that the malignant B cells in B-CLL have a markedly impaired response to the proliferation signal delivered by BCGF. The abnormal proliferative response to BCGF by CLL B cells appears to result from an impaired processing of the initial activation signals. Impaired processing of activation signals by the CLL B cells results in defective expression of cell surface receptors for BCGF by the CLL B cells. Alterations in the intensity of the initial activation signals did not improve the proliferative response to BCGF by CLL B cells. The impaired proliferative response of CLL B cells to BCGF does not appear to be the result of abnormal glycosylation of cell membrane components since neuraminidase pretreatment of CLL B cells did not improve either the proliferative response to BCGF or the expression of cell surface receptors for BCGF by CLL B cells.

Previous studies have identified intrinsic B cell defects in

CLL B cells. CLL B cells have a markedly impaired proliferative response to mitogenic signals such as PWM.[3-5] The addition of control T cells or monocytes did not improve the proliferative response of CLL B cells to PWM.[5] Decreased CLL B cell colony growth in in vitro B cell clonogenic assays has been reported.[22,23] Membrane abnormalities of CLL B cells have also been reported. CLL B cells incubated with anti-immunoglobulin reagents have abnormal dynamics of surface membrane immunoglobulin redistribution (capping).[24,25] In addition, impaired motility of CLL B cells has been described.[26,27]

The current model of normal B cell proliferation proposes that normal human B cell responsiveness to the proliferation signal of BCGF requires the expression of cell surface receptors for BCGF.[9-12] The processing of an initial activation signal by normal human B cells results in the expression of cell surface receptors for BCGF.[9-12] The presence on activated lymphocytes of receptors for specific growth factors (ie, IL-2 or BCGF) has been identified by their ability to absorb out selectively the specific growth factor activity from preparations containing the specific growth factor.[11] Other investigators have used a BCGF preparation similar to the preparation used in this study and have demonstrated that three-day, anti-μ–activated control human B cells can absorb out 55% of BCGF activity from this BCGF preparation.[11] In the current study, three-day SPA-activated control B cells absorbed out a nearly identical 54.4% of BCGF activity from the BCGF preparation. In contrast, CLL B cells consistently demonstrated a markedly impaired ability to absorb out BCGF activity from the BCGF preparation. These results suggest that CLL B cells have a defective expression of cell surface receptors for BCGF.

Cryptic or hidden expression of cell surface antigenic determinants by CLL T cells have been reported.[21] Some CLL T cells are E rosette positive but fail to react with OKT3, a monoclonal antibody specific for mature human T cells.[21] A cryptic or hidden antigenic expression of OKT3 by these CLL T cells was suggested by the finding that after neuraminidase treatment these CLL E rosette-positive, OKT3-negative T cells became reactive with OKT3.[21] The cryptic or hidden antigenic expression of the T3 antigen in these CLL T cells appears to be related to an as-yet-undefined membrane abnormality of CLL T cells that results from abnormal glycosylation of cell membrane components. The absence of any improvement in either CLL B cell proliferative response to BCGF or the expression of CLL B cell surface receptors for BCGF suggests that the abnormalities of CLL B cells reported in this study are not the result of cryptic BCGF receptor expression secondary to abnormal glycosylation of cell membrane components.

Other studies have suggested that CLL B cell dysfunction is a result of impaired differentiation.[28-31] The expression of surface membrane immunoglobulin by CLL B cells is markedly less than that expressed by more differentiated B cells.[1] It has been suggested that only normal B cells that express surface IgG are responsive to PWM.[32] Because most CLL B cell surface immunoglobulin is IgM, it has been theorized that CLL B cells may fail to respond to PWM because they lack the binding sites for PWM [5]

Murine studies have suggested that the influence of proliferation signals such as BCGF on B cells is affected by the stage of development of the target B cell.[33,34] Anti-immunoglobulin stimulation of BCL1 murine malignant B cells did not result in increased proliferation unless the BCL1 B cells had assumed a more mature phenotype before exposure to the anti-immunoglobulin.[33] Anti-immunoglobulin stimulation could not induce proliferation of normal immature murine B cells.[33]

The stage of human B cell development and differentiation during which normal B cells are responsive to BCGF is not yet known. A recent study has demonstrated that B cells responsive to BCGF may be selected by the presence on the B cell of the B2 surface antigen [35] B2 is a 140-kd glycoprotein[36] that expresses distinct binding sites for C3d[37] and for EBV.[38] B2 first appears on the cell surface after the cytoplasmic μ-chain–positive pre-B cell stage[39] and is lost when surface IgD is no longer detectable.[40] B2-positive B cells were responsive to the proliferation signals of anti-μ and anti-μ plus PHA-stimulated leukocyte-conditioned media (PHA-LCM).[35] If CLL is a disorder characterized by a clonal expansion of immature B cells,[28-31] such immature B cells may not have developed a responsiveness to the proliferation signal of BCGF.

A recent study has demonstrated some proliferative responsiveness of CLL B cells to "T cell factors."[41] However, induction of CLL B cell proliferation required activation by anti-immunoglobulin at 200 μg/mL. In that study, data on the effectiveness of more conventional concentations of anti-immunoglobulin as an initial activation signal were not presented. However, even with a 15-fold increase in the concentration of anti-μ, the stimulation index was only 3.8, comparable to results in this study in which the stimulation index was 3.1 when anti-μ at 15 μg/mL was used as an activation signal. That occasional CLL B cells may be capable of some degree of responsiveness to BCGF is suggested by the reported ability of BCGF to support the growth of some leukemic B cells in vitro for periods of up to 60 days.[11]

This study does not resolve the role of BCGF in regulating CLL B cell proliferation. It does show that the impaired responsiveness to BCGF by CLL B cells is the result of impaired expression of receptors for BCGF when CLL B cells are exposed to activation signals. The apparent arrest of CLL B cells at an immature stage of B cell development may have impact upon the responsiveness of the malignant B cell to the proliferation signal of BCGF. Because some CLL B cells may be induced to further differentiation by agents such as phorbol esters,[29-31] we have begun to investigate whether in vitro-induced differentiation of CLL B cells results in improved CLL B cell responsiveness to BCGF. Impaired responsiveness to immunoregulatory lymphokines such as BCGF may contribute to the significant hypogammaglobulinemia observed in CLL patients.

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Leukopenic Chronic T Cell Leukemia Mimicking Hairy Cell Leukemia: Association With Human Retroviruses

By C.C. Sohn, D.W. Blayney, J.L. Misset, G. Mathé, G. Flandrin, E.M. Moran, F.C. Jensen, C.D. Winberg, and H. Rappaport

We report two cases of a T cell lymphoproliferative disease not previously described, with cytologic and clinical features similar to those associated with Galton's "prolymphocytic" leukemia (PL). Our patients, like those with Galton's PL, had massive splenomegaly and minimal or absent hepatomegaly and lymphadenopathy. In contrast, however, our patients had leukopenia, as well as low percentages of leukemic cells in the peripheral blood and in the bone marrow. In splenic imprints, the nuclear chromatin pattern of most of the leukemic cells was intermediate between those of mature lymphocytes and those of lymphoblasts, and the nuclei contained single, centrally located, conspicuous nucleoli. In sections of the spleen, the leukemic cells diffusely infiltrated the red pulp in a pattern strikingly similar to that of hairy cell leukemia; however, when the leukemic cells were studied cytochemically, the cytoplasmic acid phosphatase positivity was punctate and tartrate-sensitive. The leukemic cells were sheep erythrocyte rosette-positive and expressed T cell-associated antigens. Initially, both patients responded well to therapeutic splenectomy. One patient received combination chemotherapy after splenectomy and is alive and well 24 months after diagnosis. The other patient was in complete clinical remission for one year after splenectomy and received chemotherapy at relapse. He died, however, 23 months after splenectomy, with disseminated disease. IgG antibody titers against human T lymphotropic virus type I (HTLV-I) were detected in one patient and against HTLV-II in the other. The leukemia in these patients represents a distinct clinicopathologic entity within the spectrum of peripheral T cell lymphoproliferative diseases that includes Galton's PL of T cell derivation, T cell chronic lymphocytic leukemia, T cell hairy cell leukemia, and adult T cell leukemia/lymphoma.

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THE CLINICOPATHOLOGIC and immunophenotypic heterogeneity of T cell malignancies is reflected in the growing list of T cell lymphoproliferative diseases that includes chronic lymphocytic leukemia (CLL),[1-6] hairy cell leukemia (HCL),[7-10] adult T cell leukemia/lymphoma (ATL), and peripheral T cell lymphoma,[11-18] as well as mycosis fungoides/Sézary syndrome,[19-22] and acute lymphoblastic leukemia/lymphoma.[3,23-25] Even within T cell CLL (T-CLL), several immunophenotypic and/or morphologic subtypes have been recognized,[4,5] one of which is the so-called T cell "prolymphocytic" leukemia* (PL).[5]

Galton et al[26] initially described PL as a distinct clinicopathologic variant of CLL characterized by marked lymphocytosis, massive splenomegaly, lesser hepatomegaly, and minimal or absent lymphadenopathy. Although most PLs have been shown to be of B cell derivation,[26,30-37] T-PLs[1,5,11,30,34,35,38-44] and PLs with co-expression of T and B cell markers[45] have also been described. Regardless of the immunophenotype, most reported cases of PL have conformed remarkably well to Galton's clinicopathologic criteria.

We recently encountered two cases of a leukopenic T cell leukemia in which there was massive splenomegaly but mild hepatomegaly and minimal lymphadenopathy, similar to the findings in Galton's PL and in HCL. The splenomegaly resulted from diffuse infiltration of the red pulp by lymphocytes morphologically resembling those of Galton's prolymphocytes. The presence of leukopenia distinguishes our cases from cases of Galton's PL, however, and the morphology and cytochemistry of the leukemic cells distinguish our cases from those of T-HCL. We evaluated the morphologic, immunologic, hematologic, clinical, and serologic findings in these two cases in an effort to further define the spectrum of T cell lymphoproliferative diseases.

MATERIAL AND METHODS

The presplenectomy and postsplenectomy morphological, immunologic, hematologic, and clinical material from case 1 originated from the Service des Maladies Sanguines et Tumorales (ICIG), Hôpital Universitaire Paul-Brousse, Villejuif, France. The material from case 2 was contributed by the Hematology-Oncology Section of the VA Medical Center, Long Beach, Calif, to the James Irvine Center for the Study of Leukemia and Lymphoma at the City of

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*"Prolymphocytic" leukemia as described by Galton et al[26] is a rare variant of CLL and differs from the prolymphocytic variant of acute lymphoblastic leukemia that has been described by one of us (G.M.).[27,28] We agree with Huhn[29] that the term prolymphocytic as applied to Galton's PL does not reflect our current knowledge about the stage of differentiation of the leukemic cells but, in accordance with the numerous existing reports on Galton's PL and for the sake of convenience, we will use the designation PL for the disease described by Galton.

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Morphologic Studies

Tissue for light microscopy was fixed in Bouin's solution in case 1 and in 10% buffered formalin in case 2, embedded in paraffin, and stained with hematoxylin and eosin. Touch imprints were available from the spleen in case 1 and from the spleen and splenic lymph nodes in case 2. The touch imprints as well as the blood and bone marrow smears were stained with May-Grünwald Giemsa.

Cytochemical Studies

Stains for acid phosphatase and for tartrate-resistant acid phosphatase were done on splenic imprints in case 1 and on blood smears in case 2. The α-naphthyl acetate esterase (ANAE) stain was done on blood smears in both cases. The cytochemical studies were carried out according to methods described previously.[46]

Immunologic Studies

The preparation of cell suspensions from solid tissues, the separation of mononuclear cells by Ficoll-Hypaque density gradient centrifugation, and the immunologic study of the cell suspension samples were carried out as previously described.[47,48] Neuraminidase-treated fresh sheep erythrocytes were used for the quantitation of spontaneous sheep erythrocyte rosette (sER) formation.

Cell suspensions were prepared from the spleen in case 1 and from the peripheral blood in case 2. In case 1, Leu-4 (Becton Dickinson, Sunnyvale, Calif) and T4 and T8 (Coulter Immunology, Hialeah, Fla) were used as T cell markers, and rhodamine-conjugated F(ab')2 fragments to human γ, α, μ, κ, and λ chains (Kallestad Laboratories, Austin, Tex) were used for the study of surface immunoglobulins. In case 2, T11 (Coulter Immunology) was the pan-T cell marker, and γ, α, μ, δ, κ, and λ immunoabsorbed F(ab')2 goat anti-human immunoglobulins labeled with fluorescein isothiocyanate (FITC) (Tago, Burlingame, Calif) were used for detection of surface immunoglobulins.[48] In case 2, the peripheral blood lymphoid cells were also studied by a reference laboratory (Specialty Laboratories, Inc, Los Angeles) which used OKT4 and OKT8 (Ortho Diagnostics, Raritan, NJ) as T cell markers according to standard procedures. Terminal deoxynucleotidyl transferase (TdT) activity was determined with an indirect immunofluorescence method as previously described.[47]

Serologic study for human T cell lymphotropic virus (HTLV)

Serum samples were collected, frozen, and later tested for antibodies to the HTLV by an enzyme-linked immunoabsorbent assay (ELISA) developed at Cytotech, Inc, San Diego, Calif.[49] HTLV-I was harvested from the supernatant of a cell line producing this variant (C10/MJ2, provided by Dr R. C. Gallo), HTLV-II was harvested from the 344 cell line (provided by Drs R. C. Gallo and M. Popovic), and HTLV-III was harvested from a HUT 78 cell line transfected with this virus (HUT 78/013LK). Virus-producing cells were pelleted by centrifugation, and extracellular virus was concentrated. After gradient fractionation, the purified virus was resuspended in phosphate-buffered saline (PBS) and pelleted.[49] Donor serum was diluted to 1:100 for assay. Goat anti-human IgG conjugated to glucose oxidase was used as a labeling antibody and was counterstained. The development of a green hue was determined at 405 nm in a Multiscan (Flow Laboratories, McLean, Va) automated spectrophotometer after exactly 30 minutes of incubation. Specimens were scored as positive if absorbance was 1.8 times that of a standard negative control sample for HTLV-II and 3.0 for HTLV-I and HTLV-III. Western blot analysis for HTLV-I and HTLV-III was performed as previously described.[50]

Case reports

Case 1. A 42-year-old white woman from Sicily was referred to the Hôpital Universitaire Paul-Brousse, Villejuif, France, with asthenia, low-grade fever, and a two-month history of a rapidly growing abdominal mass in the left upper quadrant. The spleen was palpable 10 cm below the left costal margin, and the liver was palpable 5 cm below the right costal margin. The patient had minimal cervical and inguinal lymphadenopathy. Peripheral blood studies revealed a WBC count of $1.9 \times 10^9/L$ with a differential count of 32% lymphocytes, 10% lymphoid cells with single conspicuous nucleoli (Fig 1a), 44% bands and neutrophils, 3% metamyelocytes, 2% eosinophils, and 9% monocytes. The Hb level was 7.2 g/dL, and the platelet count was $112 \times 10^9/L$. The bone marrow biopsy specimen was hypercellular and partially infiltrated by lymphoid cells. The bone marrow aspirate smear had originally been reported to contain 30% lymphoblasts. On review, most of these cells were interpreted as having the appearance of Galton's prolymphocytes. At splenectomy, the spleen was found to be grossly enlarged and involved by a leukemic process (Fig 2a) characterized, on touch imprints, by atypical lymphoid cells, the nuclei of which had single conspicuous nucleoli (Fig 2b). The liver biopsy specimen showed involvement by leukemia (Fig 3).

After splenectomy, the patient's WBC count slowly increased to $12 \times 10^9/L$, accompanied by an increase in the percentage of leukemic cells. There was also a transient increase in the percentage of normal-appearing monocytes. The postsplenectomy bone marrow specimen showed diffuse lymphoid leukemic infiltrations (Fig 4). The patient was treated with prednisone, vincristine, L-asparaginase, and doxorubicin, followed by maintenance therapy with 6-mercaptopurine, methotrexate, and vindesine, and by intensification therapy every three months. The agents used for intensification therapy were doxorubicin and vincristine, with either cyclophosphamide or cytosine arabinoside given alternately in successive intensification

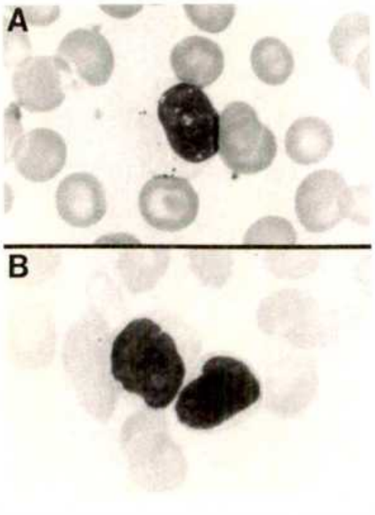

Fig 1. Peripheral blood. (A) Case 1, showing a lymphoid cell with relatively abundant cytoplasm that contains several azure granules. The ovoid nucleus has a distinct nucleolus and chromatin of intermediate coarseness. (B) Case 2. Two circulating atypical lymphoid cells show moderately abundant cytoplasm, deep nuclear indentation, and distinct nucleoli. May-Grünwald Giemsa. (Original magnification ×1360; current magnification ×816.)

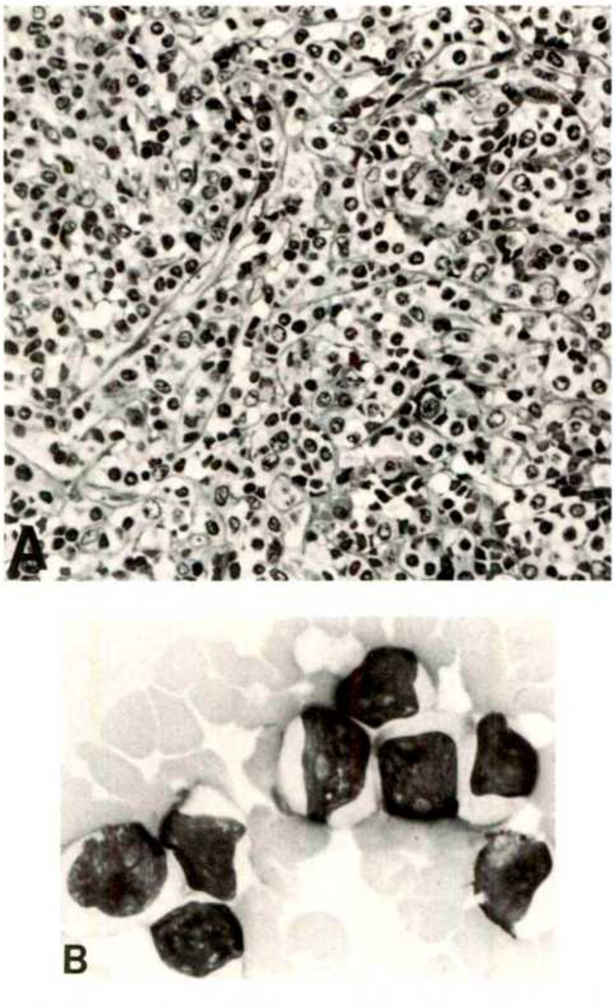

Fig 2. (A) Spleen, case 1. Red pulp showing diffuse invasion of cords and sinuses by the abnormal lymphoid cells. The abundant clear cytoplasm of many of the cells mimics the appearance of hairy cells. Hematoxylin and eosin stain: (magnification ×340.) (B) Splenic imprints exhibiting the characteristic features of the leukemic cells. The atypical lymphoid cells are characterized by a relatively abundant cytoplasm, irregular nuclear contours, distinct nucleoli, and, in most cells, a chromatin pattern intermediate between that of lymphoblasts and that of mature lymphocytes. May-Grünwald Giemsa stain. (Original magnification ×1360; current magnification ×884.)

courses. The patient has remained clinically well for 24 months, although a few leukemic cells have persisted in the peripheral blood.

Case 2. A 48-year-old black American man from Los Angeles had pancytopenia and splenomegaly at diagnosis. The spleen was palpable 10 cm below the left costal margin; there was no appreciable hepatomegaly or peripheral lymphadenopathy. The patient's peripheral blood showed a WBC count of $1.2 \times 10^9/L$, an Hb level of 9.2 g/dL, and a platelet count of $78 \times 10^9/L$. The WBC differential count showed 60% lymphocytes, including "abnormal lymphocytes" (Fig 1b), 16% neutrophils, 5% bands, 1% eosinophils, and 18% monocytes. The slightly hypercellular bone marrow biopsy specimen showed no detectable leukemic infiltration, but 8% of the cells in the bone marrow aspirate smear were lymphoid cells of intermediate size with single conspicuous nucleoli. At splenectomy, the spleen was found to be massively enlarged. Microscopic evaluation of spleen, splenic hilar lymph node, and liver biopsy specimens showed leukemic involvement. After splenectomy, the WBC count, Hb level, and platelet count returned to normal, although leukemic cells persisted in the peripheral blood. The patient was followed at monthly intervals and remained clinically well for one year.

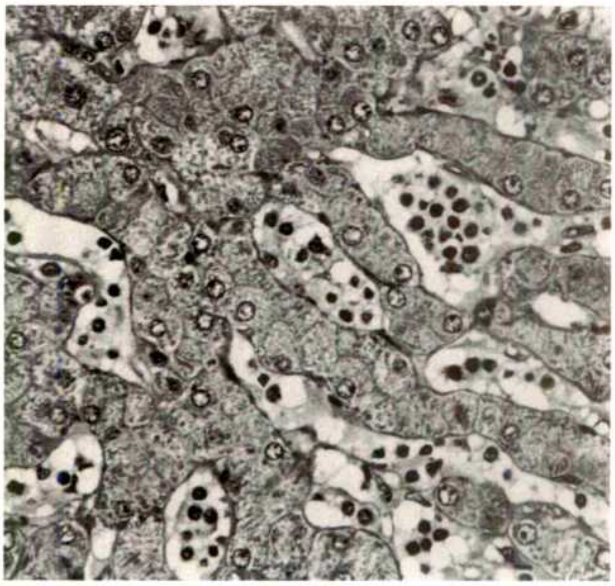

Fig 3. Liver biopsy, case 1, showing diffuse infiltration and distention of the liver sinusoids by abnormal lymphoid cells. This leukemic pattern closely resembles that observed in hairy cell leukemia. Hematoxylin and eosin. (Magnification ×340.)

Twelve months after splenectomy, the patient had a sudden, steady increase in the number of circulating leukemic cells. Biopsy specimens of bone marrow and axillary lymph nodes contained extensive leukemic infiltrates (Fig 5). Monthly five-day courses of

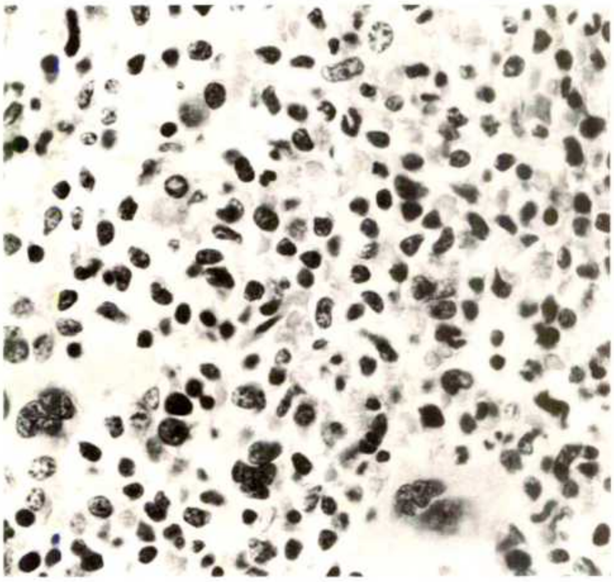

Fig 4. Bone marrow specimen, case 1 (postsplenectomy), with diffuse infiltration by lymphoid cells that exhibit variations in nuclear size and shape as well as irregularity of nuclear contours. Residual megakaryocytes are evident. Hematoxylin and eosin stain. (Magnification ×540.)

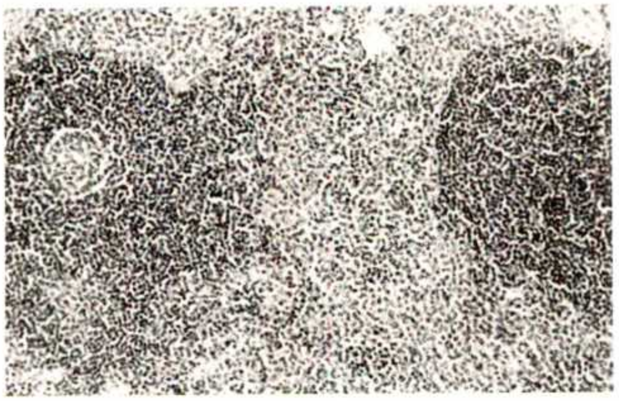

Fig 5. Lymph node, case 2, one year after splenectomy. A paracortical proliferation of abnormal lymphoid cells is in keeping with the T cell phenotype but also resembles the cellular distribution seen in lymph nodes involved by hairy cell leukemia. Hematoxylin and eosin stain. (Magnification ×110.)

chlorambucil and dexamethasone were begun when the WBC count reached $52.5 \times 10^9/L$, with almost exclusively leukemic cells; this therapy controlled but did not eradicate the leukemic cells in the peripheral blood. Five months later, the patient was again found to have an increase in the number of peripheral blood leukemic cells as well as hepatomegaly. The chemotherapy was changed to monthly cycles of cyclophosphamide, vincristine, procarbazine, and prednisone. Over the next three months, the patient's WBC count varied from 2.8 to $9.1 \times 10^9/L$, with 2% to 54% leukemic cells. The patient died of disseminated leukemia 23 months after clinical presentation. A postmortem examination was authorized and performed.

The clinical and antemortem pathologic findings in both cases are summarized in Table 1.

RESULTS

Morphologic Findings

Peripheral blood. The leukemic cells from both patients were atypical lymphocytes with relatively abundant cytoplasm. The nuclei had round to oval contours, often with one and sometimes two deep indentations. The chromatin pattern was of intermediate coarseness; usually, there was a single conspicuous nucleolus (Fig 1a and b). In case 1, the cytoplasm of many of the leukemic cells contained several azure granules (Fig 1a). No cytoplasmic granules were identified in the leukemic cells of case 2 (Fig 1b). After splenectomy, the presence of leukemic cells in the peripheral blood was more obvious because the number of circulating leukemic cells increased as the disease progressed.

Bone marrow. In case 1, the core biopsy specimen obtained prior to splenectomy showed hypercellular marrow, with persistent erythroid and megakaryocytic foci, partially infiltrated by mononuclear cells in a pattern resembling that of HCL.[51,52] The aspirate smear contained 30% atypical lymphoid cells with the same appearance as that of the leukemic cells in the peripheral blood. In case 2, there was no detectable leukemic infiltration in the initial core biopsy. The aspirate smear in this case showed only 8% leukemic cells.

In the postsplenectomy bone marrow biopsy specimens from both patients, more prominent, diffuse leukemic infiltration was evident. The lymphoid cells showed variations in nuclear size and shape and had irregular nuclear outlines (Fig 4).

Spleen. The spleens in cases 1 and 2 weighed 2,460 and 2,394 g, respectively. The cut surfaces were homogeneous and free of tumor nodules; Malpighian corpuscles were not grossly visible.

In microscopic sections, the leukemic cells filled the red-pulp cords and sinuses and encroached on or obliterated the white pulp; the pattern of involvement was strikingly similar to that in HCL (Fig 2a). The leukemic cells were of medium size and had round to oval nuclei. In routine histologic sections, the chromatin appeared less heavily clumped than in the lymphocytes of CLL. The nucleolus was often difficult to identify except when cells were examined under oil immersion. Irregularities in the nuclear contours, including deep indentations, were noted in a few of the cells. In touch imprints prepared from the spleens, the moderately coarse chromatin pattern, the single centrally located nucleolus, and the moderate to abundant pale-blue cytoplasm were clearly evident (Fig 2b).

Liver. Liver biopsy specimens from both patients contained mononuclear cell infiltrates in the sinusoids and portal areas. The pattern of infiltration, although nonspecific, resembled that of HCL (Fig 3). In the histologic sections, the cytologic features of the leukemic cells were not clearly apparent. The leukemia could not have been classified by the sections of the liver biopsies alone.

Lymph nodes. In case 2, splenic hilar lymph nodes obtained at the time of splenectomy contained leukemic infiltrates in the paracortical and medullary areas. Scattered atypical cells were identified in distended sinuses. The lymph nodes were initially interpreted as being reactive but, with the aid of the lymph node touch imprints which showed clusters of cells having single conspicuous nucleoli, they were reinterpreted as containing focal leukemic infiltrates. Focal plasma cell infiltration was also present. In the axillary lymph nodes in case 2, biopsied one year after splenectomy, sheets of leukemic cells expanded the paracortical areas and filled the sinuses (Fig 5).

Table 1. Clinical Findings

		Physical Findings at Diagnosis			Bone Marrow at Diagnosis		
					Leukemic Cells		
Cases	Age/Sex	Spleen (g)	Liver	Lymph Nodes	Biopsy	Smear	Follow-up (ms)
1	42 F	2,460	5 cm*	Minimal cervical and inguinal adenopathy	Partially infiltrated	30%	Clinically well (24)
2	48 M	2,394	Not enlarged	Not enlarged	Not detected	8%	Dead (23)

*Below right costal margin.

Postmortem examination. In Case 2, an autopsy was performed, revealing marked hepatomegaly (3,600 g) as well as moderate lymphadenopathy involving the mediastinum and the porta hepatis. Histologically, the liver contained extensive mononuclear cell infiltrates in the sinusoids and portal tracts. The lymph nodes and bone marrow were massively and diffusely involved by leukemia, whereas scattered leukemic cells were found within the interstitial tissues of the kidneys.

Immunologic and Cytochemical Findings

In case 1, immunologic studies of the cell suspensions prepared from the spleen indicated that the predominant lymphoid cell population expressed an immunophenotype consistent with that of suppressor T cells (Table 2). The majority of the cells were sER$^+$, Leu-4$^+$, T4$^-$, T8$^+$. Three percent of the cells were surface immunoglobulin (sIg) positive and had a polyclonal distribution. In case 2, the peripheral blood lymphoid cells studied at the time of lymphocytosis (one year after splenectomy) were sER$^+$, T11$^+$. They were TdT$^-$. Six percent were sIg$^+$; these cells also had a polyclonal distribution. The lymphoid cells in a presplenectomy peripheral blood specimen studied by a reference laboratory were reported as OKT4$^-$, OKT8$^-$.

In both cases, the acid phosphatase stain resulted in paranuclear granular cytoplasmic positivity which was tartrate-sensitive and often punctate in appearance. The ANAE stain also resulted in punctate, paranuclear cytoplasmic positivity, which was not diminished by sodium fluoride treatment.

Serologic Study for HTLV

Frozen serum from case 1 contained IgG antibodies to HTLV-I. The ratio of absorbance of patient serum to that of standard negative control serum was 8.4. Western blot analysis of this patient's serum was positive against HTLV-I target. Furthermore, the ratio of the HTLV-III target was 11.8, but no HTLV-III proteins were detected in the western blot system. The ELISA assay for HTLV-II was negative. Serum from case 2, obtained shortly before the patient's death, was positive for HTLV-II, with an absorbance ratio of 1.9. This specimen was negative in the ELISA assays for HTLV-I (ratio 0.9) and HTLV-III (ratio 0.96). The specimen from case 2 was also negative for HTLV-I and HTLV-III on western blot analysis.

DISCUSSION

Recent advances in immunologic techniques and the use of multidisciplinary approaches have greatly facilitated not only the identification of T cell lymphoproliferative disorders, but also the study of the state of differentiation of the neoplastic cells. Such studies have supported the assumption that chronic T cell leukemias, including Galton's PL, T cell type, are in fact derived from postthymic T cells.[3,10,43,44,53,54] Recent studies have also revealed a spectrum of clinicopathologic and immunophenotypic heterogeneity within chronic T cell leukemias, resulting in the identification of several subtypes.[1-6] We have described two cases of a chronic T cell leukemia which, we believe, represents a distinct leukopenic variant of Galton's PL.

The leukemia in the two cases reported here can be distinguished cytologically from CLL,[55] including classical T-CLL,[1] which may be associated with massive splenomegaly. This distinction can be made primarily on the basis of the chromatin pattern of the leukemic cells, which is of intermediate coarseness, and on the presence of a single conspicuous, usually centrally located nucleolus. We wish to emphasize the importance of touch imprints (Fig 2b) and of well-prepared peripheral blood and bone marrow aspirate smears for recognition of the distinctive cellular morphology of the leukemic cells. Architectural features are helpful in distinguishing leukopenic chronic T cell leukemia from lymphocytic lymphomas of intermediate or poor differentiation that present primarily in the spleen.[56] The latter display "miliary" or multifocal patterns of splenic involvement,[56] whereas in our cases the spleens were diffusely infiltrated. Likewise, the pattern of infiltration of the liver was characteristically a leukemic one (Fig 3), involving both sinusoids and portal triads without even minimal tumor formation.

The initial pancytopenia and the massive, diffuse involvement of the spleen in our two cases were findings reminiscent of those in HCL. Diffuse splenic involvement is known to occur in both B-PL and T-PL, however.[32,35] The cytologic features of the leukemic cells in our cases were those of Galton's prolymphocytes and not those of HCL; moreover, the leukemic cells exhibited punctate cytoplasmic acid phosphatase positivity that was tartrate-sensitive. Although single prominent nucleoli have been noted in the so-called "indented" type of HCL,[52] the leukemic cells in this type had notched or kidney-shaped nuclei, and their cytoplasm displayed tartrate-resistant acid phosphatase positivity. Pa-

Table 2. Hematologic, Cytochemical, and Immunologic Findings at Diagnosis

				Peripheral Blood			Immunologic Markers			ELISA HTLV			Western Blot HTLV	
Case	WBC (× 10^9/L)	Platelets (× 10^9/L)	Hemoglobin (g/dL)	(%) Lymphocytes	(%) Prolymphocytes	Cytochemistry AcP/TRAP	(%) sER	(%) (Antibody Used)	(%) sIg	I	II	III	I	III
1	1.9	112	7.2	32	10	+/−	65	92 (Leu4) 4 (T4) 80 (T8)	3	+	−	+	+	−
2	1.2	78	9.2	60	Abnormal lymphocytes*	weak +/−	67	93 (T11) 6 (OKT4) 3 (OKT8)	6	−	+	−	−	−

*An unspecified percentage of abnormal lymphocytes was noted in the initial peripheral blood smear, which was not available for review.

AcP, acid phosphatase; TRAP, tartrate-resistant AcP; +, positive; −, negative; sER, spontaneous rosettes with sheep erythrocytes; sIg, surface immunoglobulin; ELISA, enzyme-linked immunoabsorbent assay.

tients with this type of HCL generally did not have massive splenomegaly, but they tended to have marked bone marrow and lymph node involvement when first examined. The patients reported as having T-HCL or T- and B-HCL tended to have relatively slow clinical progressions.[7,8,10]

Only after splenectomy did our patients develop increases in the number of circulating leukemic cells and more extensive bone marrow involvement. At this stage of the disease, the morphologic and clinical findings were indistinguishable from those in Galton's PL. The patient who was treated with splenectomy and combination chemotherapy is clinically well 24 months after diagnosis, despite the persistence of a few circulating leukemic cells. The patient who was initially treated with splenectomy alone had a clinical remission for one year, but died nine months later with progressive disease. Treatment after relapse with chlorambucil and dexamethasone and then with cyclophosphamide, vincristine, procarbazine, and prednisone was not successful in controlling the disease.

No significant superficial lymph node enlargement was clinically evident at the outset. Minimal involvement of the splenic lymph nodes was noted in case 2 at the time of splenectomy. Enlarged axillary lymph nodes removed one year after splenectomy revealed incomplete involvement of the nodes, with a paracortical pattern of infiltration. This pattern was consistent with the T cell derivation of the neoplastic cells, but it may also be observed in HCL.

Our two cases expand the spectrum of lymphoproliferative diseases associated with various HTLV, which constitute a family of human RNA tumor viruses with T cell tropism.[57] HTLV-I, antibodies to which were found in our case 1, is associated with ATL,[58] usually involves phenotypic helper T cells, and is characterized clinically by skin lesions, lymphadenopathy, hepatosplenomegaly, hypercalcemia, and an aggressive clinical course.[11,59] In our case 1, however, the malignant cells expressed a T suppressor phenotype and clinically resembled an "aleukemic" form of lymphocytic leukemia rather than the ATL syndrome. The serologic cross-reactivity with HTLV-III seen in case 1 (i.e., ELISA positive, but negative in the western blot) is a recognized phenomenon.[60]

HTLV-II, antibodies to which were found in our case 2, is distinct from HTLV-I, based on serologic and molecular parameters, and has previously been reported in only one unusual case of T-HCL.[7,61] In contrast, our case 2, although resembling HCL with respect to its clinical presentation and to the patterns of distribution of the leukemic cells in the spleen, liver, and lymph nodes, was, in fact, a variant of chronic T cell lymphocytic leukemia in which the proliferating cells differed both morphologically and cytochemically from hairy cells.

That two clinically and pathologically similar cases were associated with antibodies against different variants of HTLV is of interest, although the significance is not clear, since the presence of these antibodies does not necessarily mean that HTLV is causally related to the evolution of the leukemia reported in our two patients. The presence of these antibodies may merely indicate coincidental infection or immunologic cross-reactivity with a previously unrecognized member of the HTLV group of viruses. Tissues involved by the leukemia were not available from our two patients for molecular or virologic studies (one patient died after the association was recognized and the other patient remains in remission). New cases of leukopenic T-PL that are identified clinically or histopathologically should be studied for evidence of HTLV-I and HTLV-II, so that the spectrum of virus-associated lymphoproliferative diseases may be defined further.

In summary, we have identified two cases of leukopenic chronic T cell leukemia with massive splenomegaly in which the leukemic cells resembled Galton's prolymphocytes. This leukemia can be differentiated from T-CLL and T-HCL on the basis of its distinctive cellular morphology. It also differs from classical T-PL by virtue of the leukopenia that is present before splenectomy. One patient (case 2) who was treated with splenectomy, died 23 months after clinical onset despite the subsequent addition of increasingly more aggressive chemotherapy. The other patient (case 1), treated with splenectomy and combination chemotherapy, is alive and clinically well after 24 months. These observations suggest the possible importance of early recognition of this distinctive leukopenic form of T cell leukemia, which, in contrast to HCL, appears to have an aggressive course similar to that in Galton's PL.

ADDENDUM

Since submission of our manuscript, three additional cases of HTLV-I-associated chronic lymphocytic leukemia have been reported,[62] although they were not leukopenic. Like our case 1, the patients were from Italy and, along with our case 1, confirm the widespread nature of HTLV-I-associated disease. These cases also suggest that Italy, particularly southern Italy, may be an endemic area for HTLV-I. Furthermore, our case 2, a black man from Los Angeles who had HTLV-II antibodies, was born and lived in the same general area as the first reported case of HCL from which HTLV-II was isolated.[61] A second such case was more recently reported by the same group of observers,[63] raising the possibility that HTLV-II-associated T cell lymphoproliferative disease may be endemic in the Los Angeles area.

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Homozygous Hemoglobin Knossos ($\alpha_2\,\beta_2 27$(B9) Ala → Ser): A New Variety of β^+-Thalassemia Intermedia Associated With δ°-Thalassemia

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Hb Knossos (β 27 (B9) Ala → Ser) is a recently discovered hemoglobin variant endowed with β-thalassemic properties(1,2) We present the first homozygous cases. The propositus, a 19-year-old man is originally from northeast Algeria, but is unrelated to other Algerians who have hemoglobin Knossos. He has a β^+-thalassemia intermedia syndrome, including microcytic, hypochromic anemia, enlargement of the spleen, and an increase in the number of reticulocytes. The reduction of β-chain synthesis is pronounced (α/non α:2.76). Whole cells containing Hb Knossos have a dramatically low oxygen affinity (P_{50}:38 mm Hg). The propositus also has homozygous δ°-thalassemia. The chromosome carrying these mutations is characterized by the DNA haplotype I.

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HB KNOSSOS (β27 (B9) Ala → Ser) in the heterozygous state has been recently recognized as the underlying abnormality in atypical β-thalassemia trait.[1,2] First identified in a family from Crete,[1] Hb Knossos was discovered soon afterwards in two families from northeast Algeria and in a family from the French West Indies.[3-5] In the heterozygous state, the Hb Knossos mutation causes "normal Hb A_2" β-thalassemia trait and, when associated with a "high Hb A_2" β-thalassemia trait or a Lepore fusion gene, generates a picture of β-thalassemia intermedia[1,3,5] The low amount[1,4] or absence of Hb A_2[5] has suggested that the β^{Knossos} gene is flanked by a δ°-thalassemic gene, at least in persons of mediterranean origin. We present the two first homozygous persons who have β^+-thalassemia intermedia associated with δ°-thalassemia.

MATERIALS AND METHODS

Case report. The propositus (II.1, Fig 1B), is a 19-year-old Algerian who has lived in France since 1982. He was born in Rabta, 80-km west of Setif in northeast Algeria. His parents are not consanguineous, but both originate from Rabta. The present family is apparently unrelated to the two Algerian families in which Hb Knossos was previously described.[3-5] Hematological abnormalities were discovered during evaluation for an unrelated orthopedic problem. Mild cutaneous pallor was noted. The spleen extended 1 cm below the costal margin. Radiological evaluation of the skeleton revealed abnormalities of the right humerus and the left femur, a C5 hemivertebra, and a L5 spina occulta, but the characteristic stigmata of thalassemia were absent. The red cell indices were RBC, 5.14×10^{12}/L, Hb, 10,1 g/dL; MCV, 52 fL (serum iron 4.5 μmol). Anisocytosis and target cells were present.

The sister (II.4) of the propositus is also a homozygote for Hb Knossos. She is 8 years old and asymptomatic. Neither icterus nor splenic enlargement were present. However, she had moderate anemia (Table 1). The parents and two brothers who were studied show a hematological picture characteristic of heterozygous Hb Knossos[1,3,4] (Table 1).

Methods. Hemoglobin analysis was performed by isoelectric focusing (IEF) according to Basset et al,[6] or on agarose gels provided by Sebia for Hb A_{1C} determination. Globin chains were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of Triton X-100 and 8 mol/L of urea.[7] Gels were scanned at 570 nm. Hb A_2 was assayed using microchromatofocusing[8] and Hb F by the procedure of Betke et al.[9] γ-Chains were studied by Triton-urea PAGE[7] after alkali precipitation of Hb A and/or Knossos. α^A and β^{Knossos} chains from homozygotes were separated by ion exchange chromatography.[10] The β-chain was subjected to aminoethylation and to tryptic digestion.[11] The peptides were analyzed by fingerprinting[11] or reverse-phase high-performance liquid chromatography (HPLC) on a μ Bondapak C18 column (Waters, Milford, Mass) in a LKB system (Bromma, Sweden), using an increasing gradient of acetonitrile in 0.01 mol/L of ammonium acetate (pH 5.7), with a flow rate of 1.50 mL/min.[12,13] Amino acid composition of abnormal peptide βT3 was performed in a Biotronic amino acid analyzer. In vitro globin chain synthesis was carried out essentially according to Lingrel and Borsook.[14] Oxygen dissociation curves were performed in a Hemoscan differential spectrophotometer.

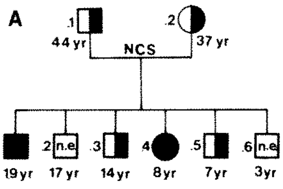

Fig 1. Pedigree of the family studied: □, β^A gene; ■, β^{Knossos} gene. Ages of family members are shown.

DNA was obtained from leukocytes as described[15] and was digested (10 to 15 μg) in the presence of restriction endonucleases, using conditions recommended by the manufacturers. DNA fragments were separated in 0.8% agarose gels, transferred to nitrocellulose filters,[16] and hybridized with ^{32}P-labeled probes. Four probes were used to recognize the seven polymorphic sites described by Orkin et al[17]: (a) a 1.3-(kb) *Bam* HI/*Eco*RI ϵ gene fragment in pBR 328; (b) a 0.8-kb *Pst* I/*Eco*RI Aγ IVS II in PSP 65; (c) a 1.7-kb $\psi\beta$ *Bgl* II/*Xba* I fragment in pBR 328; and (d) a 0.95-kb *Bam* HI/*Eco*RI β IVS II in pBR 322.

RESULTS

In the untransfused propositus (II.1), Hb A_2 was absent (Table 1, Fig 2a and c). Hb A was replaced by a component focusing slightly more cathodically (Fig 2a). This change became more apparent when IEF was carried out in agarose

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Table 1. Red Cell Indices and Hemoglobin Parameters

Index (Parameters)	I.1	I.2	↗II.1	II.3	II.4	II.5
RBC (× 10^{12}/L)	5.84	4.75	5.32	4.86	5.24	5.07
Hb (g/dL)	16.0	11.7	10.1	11.1	9.5	11.8
MCV (fL)	79.5	74.4	58.4	70.2	58.3	71.5
MCH (pg)	27.3	24.7	19.1	22.8	18.1	23.4
Reticulocytes (%)	4.80	1.80	6.00	1.0	2.80	1.50
Serum iron (μmol/L)	18	6	15	16.5	20.8	—
Hb Knossos (%)*	—	—	96.6	—	95.3	—
$\beta^{Knossos}/\beta$ (%)†	37.5	37.6	—	37.1	—	38.1
Hb F (%)	0.38	0.81	2.30	1.60	4.72	2.43
Gγ (%)†	41.1	20.0	21.1	36.5	26.2	39.7
Hb A_2, (%)‡	1.94	1.44	0.00	2.24	0.00	2.39
α/non α	1.69	1.35	2.76	—	—	—
P_{50}, mm Hg§	33.5	35.5	38.0	—	—	—
$\bar{n}$ Hill§	2.56	2.52	2.62	—	—	—
2,3-DPG (μmol/g Hb)§	14.12	13.88	13.04	—	—	—

II.1 and II.4 are homozygotes; ↗, propositus. The reason for the elevation of the reticulocyte count in I.1 is unknown.

*Determined by chromatofocusing.

†Determined by scanning of polyacrylamide-urea-Triton gels.

‡Normal values 2.69% ± 0.62% (n = 352).

§Normal values (n = 15): P_{50} = 27.20 ± 1.57 mm Hg; $\bar{n}$ = 2.69 ± 0.11; 2,3-DPG = 12.83 ± 1.90 μmol/gHb.

gels (Fig 2b). Electrophoresis in a polyacrylamide gel in the presence of Triton X-100 ascertained the existence of a β-chain variant in the homozygous state. Hb F was slightly increased with a $^{G}\gamma$ percentage of 21.1%. A similar picture was found in the second homozygote (II.4) (Table 1).

Further studies were carried out in the propositus. The in vitro synthesis of β-chains was depressed (α/non α = 2.76). P_{50} was dramatically increased (38 mm Hg) and $\bar{n}$ remained normal (Fig 3). Fingerprints of the abnormal β-chain appeared normal (Fig 4a), but high-performance liquid chromatography (HPLC) profiles allowed recognition of an abnormal peptide (Fig 4b). Amino acid analysis ascertained that the abnormal peptide was T3, with a serine residue in the place of an alanine residue (Table 2). These data identified the abnormal hemoglobin as Hb Knossos (β27(B9) Ala → Ser). The non-α globin gene cluster carried haplotype I (+ − − − − + +), the most frequently encountered haplotype among persons of mediterranean origin.[18]

In the parents and the two heterozygous brothers (II.3 and II.5), Hb A_2 was normal or slightly decreased (Table 1). Conventional IEF on polyacrylamide gel analysis failed to detect Hb Knossos revealed on agarose gel. Electrophoresis of Triton-urea gels showed that the precentage of the $\beta^{Knossos}$ chain in heterozygotes was remarkably homogeneous, but substantial variations of the $^{G}\gamma$ levels were found. However, since it can reasonably be assumed that the paternal and maternal Knossos chromosomes 11 are identical, the $^{G}\gamma$ percentages suggest that II.3 and II.5 have inherited the

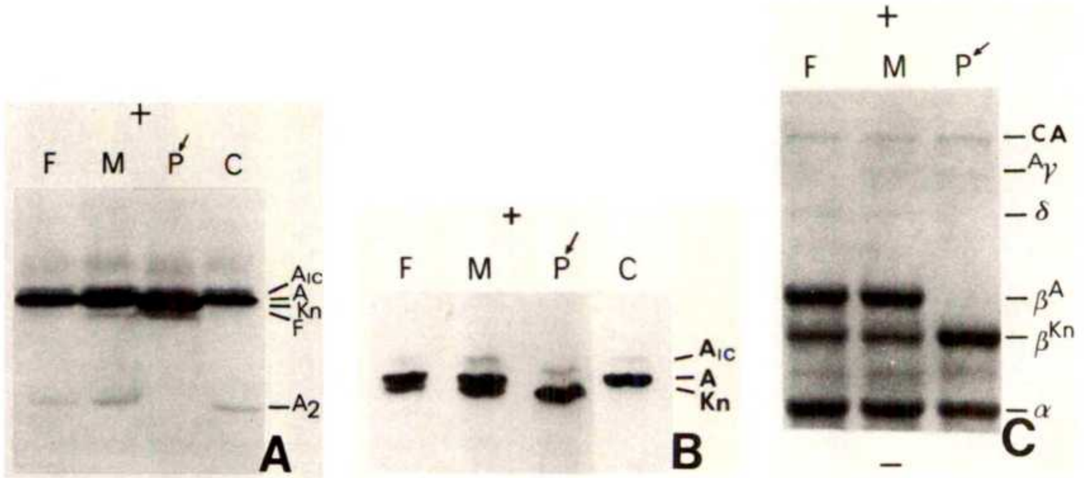

Fig 2. Isoelectric focusing and electrophoretic analyses: P, propositus; F, father; M, mother; C, control; (A) IEF on polyacrylamide gel electrophoresis[8]; (B) IEF on agarose gel; (C) electrophoresis on polyacrylamide gel in the presence of urea-Triton X-100.[7] CA, carbonic anhydrase.

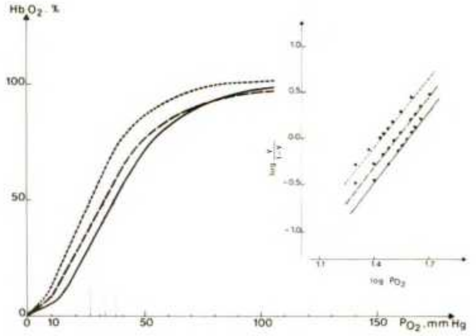

Fig 3. Oxygen dissociation curves: (· · ·) control ($\bar{n}$ = 2.56); (--) father; (—) propositus.

normal chromosome 11 from their father. In the parents, the α/non-α ratios and the P_{50} were found to be moderately increased.

DISCUSSION

We described the third Algerian family with Hb Knossos.[3-5] To our knowledge, the propositus and her sister are the first recorded homozygous cases. The similarity of haplotypes associated with the Greek[18] and the Algerian mutations suggests that the same chromosome is involved among persons of northern and southern mediterranean origin, in contrast to the mutation found in blacks and in Creole people that is not flanked by a δ°-thalassemia gene and is associated with another haplotype.[19] Although it is not known on which side of the Mediterranean basin hemoglobin Knossos first arose, it is worth noting that the three Algerian families carrying this hemoglobin originate from a 200-km² region in northeastern Algeria. Given the apparently low frequency of the β^{Knossos} gene,[4] the detection of homozygotes in independent families would probably require a large scale screening of β^{+}-thalassemia intermedia patients in this part of Algeria.

The difficulty in detecting Hb Knossos must be emphasized. All families described to date that display Hb Knossos were discovered because of other β-chain abnormalities, β-thalassemia, Hb Lepore, or Hb S, facilitated demonstration of Hb Knossos. The present family was recognized by study of a homozygote. The association of a microcytosis with normal serum iron and an inconstantly decreased Hb A_2 usually suggests a mild form of α-thalassemia, but Hb Knossos should be considered and the appropriate screening technique should be used.

The present study of Hb Knossos homozygotes confirms the presence of a δ°-thalassemia gene in cis to the β gene mutation previously suspected[1,4] or demonstrated[5] among persons of mediterranean origin. δ°-Thalassemia has been described in different populations.[20-23] In homozygous cases,[22] a distinct δ°-thalassemia gene must be involved since the hematological data are not consistent with a homozygous Hb Knossos. No large deletion was found to be associated with the δ°-thalassemia gene cis to the β^{Knossos} gene.[5] Similarly, previous studies on δ°-globin genes have shown nondeletion defects.[23,24]

These two cases of homozygous Hb Knossos may be

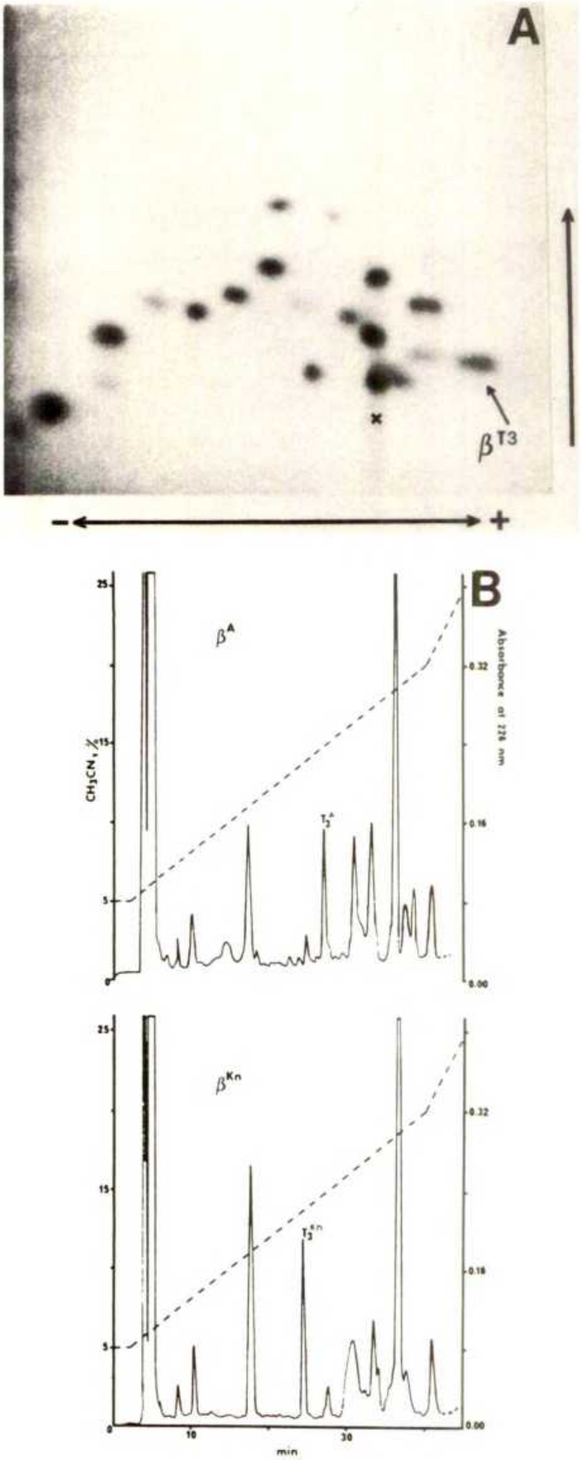

Fig 4. Peptide analysis: (A) tryptic digestion fingerprints; (B) high-performance liquid chromatography profiles of a control (top) and the propositus (bottom).

Table 2. Amino Acid Composition of the $\beta T3^{Knossos}$ Peptide

Amino acid	Normal Values	$\beta T_3^{Knossos}$
Asx	2	1.70
Glx	2	2.30
Ser	0	0.92
Gly	3	2.92
Ala	1	Traces
Val	3	3.00
Leu	1	0.85
Arg	1	0.77

compared to Hb E disease. Both Hb Knossos and Hb E reflect mutations that also contribute to abnormal mRNA splicing.[18,25] The 8-year-old child (II.4) has significant anemia but is asymptomatic. The propositus had comparable anemia but the spleen was enlarged. Perhaps this condition may evolve into a more serious clinical syndrome as has been the case for the patient with Hb Knossos and Hb Lepore whom we recently described.[5] Homozygous Hb Knossos appears to be more severe than Hb E disease, which may reflect a more pronounced imbalance in globin chain synthesis (2.76 v 1.43 to 2.30 (n = 8)) (see refs. 26–28). Decreased oxygen affinity should reduce the clinical severity. Paradoxically, the β-thalassemia-$\beta^{Knossos}$ association is less severe[1,3] than the β-thalassemia-β^{E} association.[29]

The present report describes the two first cases of homozygous Hb Knossos in an Algerian family. This genotype produces the phenotype of β^{+}-thalassemia intermedia. The most salient features found in the propositus are the marked imbalance in globin chain synthesis, the reduced affinity of Hb Knossos for oxygen, and the association of the $\beta^{Knossos}$ gene with a δ^{0}-thalassemia gene and DNA haplotype I.

ACKNOWLEDGMENT

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Defective Erythroid Progenitor Differentiation System in Congenital Hypoplastic (Diamond-Blackfan) Anemia

By Jeffrey M. Lipton, Michele Kudisch, Rachel Gross, and David G. Nathan

To explore the etiology of congenital hypoplastic or Diamond-Blackfan anemia (DBA) we investigated in vitro erythropoiesis in nine patients. Of the nine, seven were clinically responsive to prednisone. Four were infants evaluated at the time of diagnosis. Six were never or were only minimally transfused. Those for whom prednisone had been prescribed had discontinued the drug a minimum of five months prior to study. The bone marrows of these nine patients were compared with those of hematologically normal individuals and with those of four patients with transient erythroblastopenia of childhood (TEC) whose erythroid aplasia was as severe as that of the patients with DBA. Using the plasma clot semisolid culture technique to enumerate erythroid progenitors and to evaluate the growth characteristics of the colonies to which they give rise, we concluded that at the onset of DBA: (a) erythroid progenitor frequency does not correlate with the degree of anemia and erythroblastopenia; (b) erythroid progenitor differentiation may in some cases be abnormally insensitive to crude preparations of erythropoietin; and (c) progenitor erythropoietin insensitivity in vitro does not necessarily indicate prednisone insensitivity in vivo. Thus, DBA does not appear to be solely the result of deficient formation of erythroid progenitors but is, in addition, a disorder that is due to defective progenitor differentiation in vivo.

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THE PATHOPHYSIOLOGY of congenital hypoplastic anemia or Diamond-Blackfan anemia (DBA) has remained obscure for >40 years. This rare macrocytic aregenerative anemia of childhood is characterized by moderate to severe anemia usually beginning in infancy. Other cell lines appear less affected, although mild neutropenia is occasionally observed and thrombocytosis is common. The bone marrow is characterized by normal cellularity with erythroid hypoplasia. Proerythroblasts are sometimes present, but erythroid differentiation is markedly diminished overall. Seventy-five percent of patients respond to prednisone, sometimes in vanishingly small doses, with complete or partial resolution of their anemia. Even in responsive patients the macrocytosis rarely disappears. The diagnosis and treatment of DBA has been thoroughly reviewed.[1,2]

In vitro culture of marrow mononuclear cells in semisolid media permits assessment of the apparent frequency of committed erythroid progenitors and, to a limited extent, their function.[3-5] By means of these culture techniques, the erythroid failure in DBA has been attributed to a severe[6] or moderate[7] deficiency of erythroid progenitors. This deficiency has been ascribed to failure of progenitors with pluripotentiality to differentiate into committed erythroid burst-forming units (BFU-E), the first step in the erythroid differentiation pathway. The studies that lead to these conclusions were performed on the marrows of prednisone-treated or multiply transfused older patients, however. Even in these studies, a qualitative dysfunction of erythroid progenitors, as manifested by a higher than normal erythropoietin requirement for terminal differentiation in addition to a quantitative deficiency of progenitors, was obtained. Further evidence for erythropoietin insensitivity was later provided by Chan and co-workers.[8,9] Therefore, we examined the marrows of newly diagnosed patients with DBA to determine the characteristics of DBA erythroid progenitor differentiation in the absence of the complications of therapy or the influence of disease chronicity. Five patients were examined at or near diagnosis. The study was carried out within two years of diagnosis in three of the remaining four patients, and all four had discontinued prednisone a minimum of five months prior to evaluation. Five of the patients were studied in infancy, and three more were studied within the first four years of life. One evaluated patient was an adult who had not been regularly transfused.

The results of this investigation of newly diagnosed patients have altered our concept of the pathophysiology of the disease. Although erythroid progenitor numbers as measured by their differentiation into colonies were lower than normal, particularly in steroid-resistant patients, we found them to be in excess of what was expected based on a precursor product relationship of progenitors to bone marrow erythroblasts and peripheral blood reticulocytes and much higher than what was reported in older, transfusion-dependent patients.[6] However, insensitivity of DBA progenitor differentiation to crude erythropoietin in vitro was found in both of the steroid-nonresponsive patients. Two of the steroid-responsive patients demonstrated a degree of crude erythropoietin insensitivity comparable to that of the nonresponsive patients, whereas three showed a mild to moderate degree of insensitivity and two had a normal response to crude erythropoietin in the system we use. On the basis of these results, we suggest that, at least at the onset of DBA, the initial stages of erythroid progenitor maturation proceed essentially normally from multipotent to committed erythroid progenitors. However, although the defect in three of the steroid-responsive patients was minimal, most patients do manifest an abnormality in the terminal differentiation of erythroid progenitors characterized by an insensitivity to crude erythropoietin. The extent of this erythropoietin insensitivity in vitro is quite variable and does not correlate with a

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Table 1. Clinical Characteristics of DBA Patients

Patient	Age at Diagnosis	Age at Time of Study	Peripheral Blood (at Presentation)		Percentage of Bone Marrow Nucleated Cells		Prednisone Responsive	Transfusions Prior to Study (No)
			Hemoglobin (g/100 mL)	Reticulocytes (%)	Proerythroblasts (%)	Other Erythroblasts (%)		
E.D.	2 mo	2 mo	8.3	0.6	0	1 BN	Yes	Yes (1)
M.H.	2 mo	2 mo	5 8	1 6	2	0	Yes	No
K.G.	3 mo	3 mo	3.9	1.5	0	0	Yes*	No
J.F.	2 mo	1 yr, 8 mo	6 6	0.3	0	3 BN, 2 ON	Yes	No
J.C.	1 mo	8 mo	5.3	0.0	7	1 BN	Yes	Yes (5)
H.S.	4 mo	2 yr, 8 mo–3 yr, 9 mo	3.0	0.2	1	2 BN, 3 PN	Yes*	Yes (MT)
P.M.	2 yr, 2 mo	23 yr	6.6	0.0	0	0 3 PN	Yes	Yes (MT)
R.C.	1 mo	1 mo–1 yr, 10 mo	6.2	0.2	2	0	No	No
J.D.	2 mo	1 yr, 2 mo	2.8	0.0	0	0	No	Yes (MT)

DBA, Diamond-Blackfan anemia; PE, proerythroblast; BN, basophilic normoblast; PN, polychromatophilic normoblast; ON, orthochromatic normoblast; MT, multiple transfusions.

*Although hematologically responsive to prednisone, untoward side effects such as linear growth retardation, weight gain, and hypertension necessitated prednisone withdrawal and transfusion dependence.

response to prednisone in vivo. Whether these findings are the result of an intrinsic progenitor defect or some other abnormality in the system that regulates terminal erythroid differentiation remains to be determined.

MATERIALS AND METHODS

Patients Bone marrow was obtained after informed consent was received from nine patients who fulfilled the diagnostic criteria for DBA.[1] The volume of all bone marrow aspirates was ~2 ml. The cell count after Ficoll-Hypaque density centrifugation[10] was in all cases >40 × 10^6 mononuclear cells per aspirate. Thus, contamination by peripheral blood mononuclear cells and dilution of progenitors by these cells was considered to be <10%. The timing of the studies with relationship to age, diagnosis, and treatment is shown in Table 1. Three of the steroid-responsive patients were initially studied at diagnosis, two were studied prior to administration of red cell transfusions, and one was studied after a single transfusion A fourth responsive patient was evaluated 18 months after diagnosis. She first received a brief course of androgens, followed by prednisone therapy with no transfusions. Two of the three other steroid-responsive patients were periodically transfused for seven months and 28 months, respectively, prior to this study. The remaining responsive patient, the only adult studied in this investigation, was transfused initially at diagnosis and received transfusions at the time of reinstitution of prednisone therapy after multiple elective cessations over a 20-year period. Of two steroid-nonresponsive patients, one was studied initially at diagnosis and before she received a transfusion, whereas the other was transfused approximately monthly for the 12 months prior to evaluation. Of the five patients previously treated with prednisone, all had discontinued the drug for a minimum of five months prior to evaluation. In addition to the normal marrows with which these studies of DBA marrow were compared, we also evaluated the marrows of four patients with transient erythroblastopenia of childhood or TEC. The patients were 12, 16, 19, and 21 months of age, respectively. They all had reticulocyte counts of <1.0% with hemoglobins of 4.0, 4.3, 6.1, and 4.0 g/dL, respectively. Spontaneous recovery of erythropoiesis occurred within one month of evaluation of all four cases.

Cell culture. Bone marrow low-density mononuclear cells prepared by Ficoll-Hypaque density centrifugation were plated at a cell density of 5 × 10^5/mL in the plasma clot culture system previously described[11] and modified.[12,13] The culture system contained from 0.1 U/mL to 4 U/mL of erythropoietin from various sources.* In some experiments, burst-promoting activity (BPA), the supernatant of the Mo cell line[14] kindly provided by Dr David Golde, was substituted for NCTC-109 (Microbiological Associates, Bethesda, Md) as 5% of the plasma clot culture. We find this source of crude BPA to be comparable to that derived from leukocyte-conditioned medium.

Clotting was initiated by the addition of 0.1 mL of NCTC-109 containing 1 U of grade I bovine thrombin (Sigma Chemical Co, St Louis). The 1.0 mL of clotting mixture was dispensed in 0.1-mL aliquots into 0.2-mL microtiter culture wells (Linbro plates, Linbro Scientific Co, New Haven, Conn) and incubated under 5% CO_2 in high humidity. The plasma clots were incubated for up to 14 days. The clots were fixed and stained as described by McLeod and co-workers.[11] Erythroid colonies in three to six clots were counted, and the results were expressed as the mean and SEM of the number of erythroid colonies per 10^5 cells plated. CFU-E were enumerated on day 7, and BFU-E were enumerated and scored according to size from 1+ to 4+ on days 11 through 14 as previously described.[13] Control marrows were obtained from hematologically normal individuals from three months of age to 50 years of age. Two were less than one year of age. The control studies were carried out during the time period in which this study was performed, frequently simultaneously, using identical tissue culture techniques.

RESULTS

Progenitor-derived colony enumeration. Previous data acquired in patients with long-standing DBA, of whom half were steroid resistant, suggested that the disorder in this group is characterized by a block in progenitor maturation at some point between the multipotent myeloid progenitor and the BFU-E stage of differentiation.[6] To evaluate this finding in the patients studied, erythroid progenitor-derived colonies were enumerated in plasma clot at 2 U/mL of erythropoietin. A degree of marrow erythroid aplasia far in excess of that predicted by the usual precursor product relationship of CFU-E to marrow erythroblasts provides strong evidence that in vivo progenitor maturation is relatively intact from the multipotent progenitor to the CFU-E stage of differentiation, and that the in vivo defect in DBA lies in the inability of CFU-E to differentiate beyond that stage. As shown in Fig 1, the mean and 1 SD of the frequency of CFU-E–derived colonies in DBA was lower than normal; however, normal frequencies were found in four of seven steroid-responsive patients. (The SEM for individual patients and controls was in the range of ±15% to 25%, in agreement with previous work using these culture techniques.[6]) The progenitor frequencies in the three remaining steroid-responsive patients, although low, were far in excess of that expected for the degree of reticulocytopenia. The cultures of marrows of the two steroid-nonresponsive patients provided relatively poor colony growth. CFU-E colony expression in the steroid-nonresponsive patients fell below 1 SD of the mean for the nine DBA patients. However, addition of a source of BPA to the latter cultures significantly enhanced their CFU-E–derived colony expression, suggesting that CFU-E are present in the marrow in sufficient numbers but that, in contrast to the less severely affected steroid-responsive patient, standard culture techniques do not overcome the block in differentiation to hemoglobin-containing cells unless BPA is added. The addition of BPA enhanced CFU-E–derived colony numbers in all five of the DBA marrows to which it was added. In contrast, the frequency of CFU-E–derived colonies was enhanced by BPA only in one apparently normal marrow. The marrow from this individual provided the only "normal" study in which CFU-E–derived colony frequency, in the absence of BPA, was below the normal range. Measurements of BFU-E–derived colony frequencies provided similar results. Three of the seven steroid-responsive patients had normal colony frequencies. BFU-E colony frequency was markedly increased in two of these patients and was moderately increased in a third steroid-responsive patient in the presence of BPA. Neither of the steroid-nonresponsive patients produced normal BFU-E–derived colony frequencies, and colony size was persistently deficient (1+ instead of 3 to 4+) in five of the cultures of the marrows of steroid-responsive patients and both steroid-nonresponsive patients. The significant enhancement of CFU-E–derived colony expression even for prednisone-resistant patients in the presence of BPA and BFU-E–derived colonies that were considerably smaller than those derived from normal controls strongly suggest that the failure of erythropoiesis results primarily from dysfunction of the terminal differentiation of erythroid progenitors rather than from their absence.

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*The erythropoietin sources used in this study were: Sheep plasma, Connaught, Step III, Connaught Medical Research Laboratories, Willowdale, Ontario, Canada (Lot nos. 3029-1, 3031-1, 3032-1, 3039-1, 3042-1, 3076-1, and 3081-1), specific activity (sp. act.) 3 to 5 U/mg of protein; human urinary, National Heart, Lung and Blood Institute (NHLBI) (H-36-TaLSL), sp. act. 28 U/mg of protein; human urinary NHLBI (Arg-8-TaLSL), sp. act. 27 U/mg of protein; human urinary, NHLBI (E-8-39LSL), sp. act. 28 U/mg of protein; human urinary, Dr John Adamson, Seattle, sp. act. 30 U/mg of protein; human urinary, Dr Arthur Sytkowski, Boston, sp. act. 96 U/mg of protein; and human urinary, Toyobo Co, Ltd, Osaka, Japan, (Lot no. 1668), sp. act. 49 U/mg of protein.

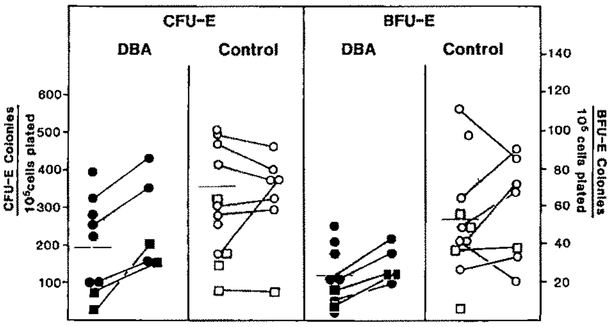

Fig 1. Erythroid progenitor-derived colony frequency in patients with Diamond-Blackfan anemia (DBA). CFU-E- and BFU-E-derived colonies are plotted on the left and right vertical axes, respectively, as colonies per 10^5 bone marrow cells plated. Steroid-responsive (●) and steroid-nonresponsive (■) patients with DBA are compared with hematologically normal controls (O) and patients with transient erythroblastopenia of childhood (TEC) (□). Shaded areas represent 1 SD from the mean for each group. TEC patients are not included in the mean colony counts and SD for the controls. Effect of burst-promoting activity (BPA) on colony frequency is shown on the horizontal axis.

Erythropoietin sensitivity of progenitor differentiation. There appears to be a complex system involving hematopoietic growth factors and cell–cell interactions that regulate the terminal differentiation of erythroid progenitors.[15] To gain further evidence of dysfunction of this erythroid progenitor differentiation system, we measured the crude erythropoietin requirement for in vitro DBA progenitor differentiation. To do so, we enumerated progenitor-derived colonies as a function of the erythropoietin concentration in the cultures. From these data, an erythropoietin dose-response curve was generated (Fig 2), and the [Epo]½max, that concentration of erythropoietin at which half the maximal number of progenitor-derived colonies are obtained, was determined. The abnormal dose-response curve shown in Fig 2 is one of two patient curves that do not approach a plateau level for erythroid progenitor-derived colony expression at or below an erythropoietin concentration of 4 U/mL. In this case, greater colony expression at a higher erythropoietin concentration would shift the [Epo]½max to an even higher value. As summarized in Fig 3, of the seven steroid-responsive patients, two had an abnormally high [Epo]½max for BFU-E and CFU-E indicative of

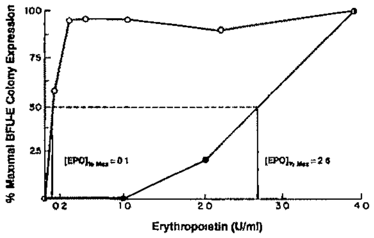

Fig 2. Representative erythropoietin dose-response curves in two patients with Diamond-Blackfan anemia (DBA) in the absence of exogenous burst-promoting activity (BPA). The percentage of maximal BFU-E colony expression on the vertical axis is plotted as a function of erythropoietin concentration from 0 to 4.0 U/mL on the horizontal axis for a patient with normal in vitro erythropoietin sensitivity (O—O) and a patient with low in vitro erythropoietin sensitivity (●—●). The concentration of erythropoietin at which there is 50% maximal BFU-E colony expression is defined as the half maximal erythropoietin concentration or [Epo]½max.

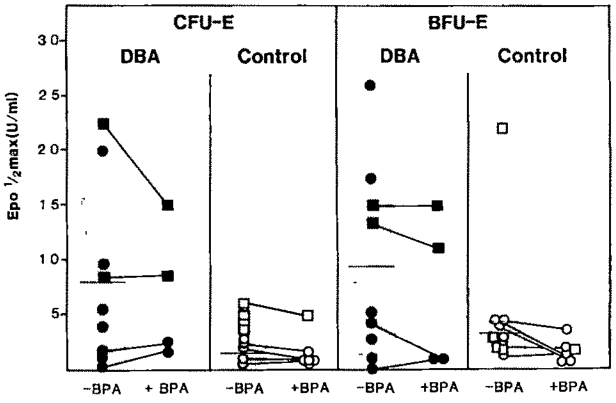

Fig 3. Half maximal erythropoietin concentration, [Epo]½max, the concentration of erythropoietin at which there is 50% maximal erythroid colony expression, in patients with Diamond-Blackfan anemia (DBA). The [Epo]½max for BFU-E and CFU-E plotted on the vertical axis for steroid-responsive (●) and steroid-nonresponsive (■) patients with DBA are compared with hematologically normal controls (○) and patients with transient erythroblastopenia of childhood or TEC (□). The shaded areas represent 1 SD from the mean for each group. TEC patients are not included in the mean colony counts and SDs the controls. The effect of burst-promoting activity (BPA) on the [Epo]½max is shown on the horizontal axis.

in vitro insensitivity to erythropoietin. Thus, five patients with steroid-responsive DBA had no evidence of a high erythropoietin requirement when compared with normal subjects and patients with TEC as described below. However, of these five, three had small (1+) BFU-E–derived colonies at two units of erythropoietin per milliliter. Both of the steroid-nonresponsive patients had an elevated [Epo]½ max and small progenitor-derived colonies. Although evaluated in only two abnormal patients, the presence of BPA did not normalize the [Epo]½max in steroid-resistant patients. In summary, an in vitro defect in the terminal differentiation of erythroid progenitors measured by an elevated [Epo]½max could be demonstrated in two of seven prednisone-responsive and in both prednisone-nonresponsive patients. Five steroid-responsive patients had progenitor differentiation that exhibited a normal [Epo]½max when compared with normal and TEC progenitors. Three of these patients and both steroid-resistant patients had small BFU-E–derived colonies. Two of the steroid-responsive patients exhibited normal erythropoietin sensitivity as measured by [Epo]½max and colony size. Table 2 summarizes DBA progenitor-derived colony enumeration and erythropoietin dose response data.

Progenitor colony enumeration and erythropoietin sensitivity in transient erythroblastopenia of childhood (TEC). To determine whether the findings in DBA were merely due to some unexpected and ill-defined result of severe anemia and erythroid hypoplasia, we pursued similar studies in patients with TEC, a spontaneously resolving red cell aplasia which initially is frequently indistinguishable from DBA.[2,16,17] Both syndromes are characterized by anemia, reticulocytopenia and elevated plasma erythropoeitin activity, and the absence of erythroid precursors in the marrow, but TEC is not familial and there are no associated congenital abnormalities. Erythroid failure in many TEC patients appears to be due to an IgG inhibitor directed against erythroid progenitors.[18,19] In agreement with the studies of Dessypris et al,[19] we confirm that progenitor colony enumeration in TEC yields variable results (Fig 1). One patient had normal CFU-E- and BFU-E–derived colony frequencies. Two patients had low numbers of CFU-E- and normal BFU-E–derived colony numbers, and a fourth TEC patient had low BFU-E and CFU-E colony frequencies. The presence of BPA had no effect on progenitor-derived colony expression in the one TEC patient so studied. The BFU-E [Epo]½max values in the three TEC patients with normal BFU-E-derived colony numbers were within the normal range. BFU-E colony morphology was normal (4+) in those three cases. The [Epo]½max for BFU-E–derived colonies in one patient was elevated, indicative of erythropoietin insensitivity. BFU-E–derived colonies in this case were small (1+). All TEC patients had mild to moderate elevations in [Epo]½max for CFU-E–derived colonies Thus, in the patients with TEC in whom anemia, reticulocytopenia, and bone marrow erythroid aplasia were comparable to that observed in DBA, erythroid progenitor behavior was different. BFU-E–derived colony frequency, morphology, and response to erythropoietin was normal in three of four patients, whereas CFU-E, although usually reduced in frequency, had an essentially normal in vitro response to erythropoietin. If one considers the minimal elevation in CFU-E [Epo]½max in TEC to be in some way related to the degree of erythroblastopenia observed in both TEC and DBA, both steroid-nonresponsive and two steroid-responsive patients clearly have a progenitor differentiation abnormality as measured by an abnormal [Epo]½max. These observations support the contention that the abnormal in vitro colony frequencies, morphology, and response to crude erythropoietin when observed in patients with DBA are actually due to defects in progenitor differentiation and are not caused by marrow erythroid hypoplasia and elevated plasma erythro-

Table 2. Progenitor Enumeration and Half Maximal Erythropoietin Concentration [(Epo)½ max] of Erythroid Progenitors in DBA

Patient	Steroid Responsive	BPA Added	Colonies/10^5 Cells Plated ± SEM* at 2 U Epo/mL		[Epo]½ Max (U/mL)	
			BFU-E	CFU-E	BFU-E	CFU-E
E D.	+	–	32 ± 5	267 ± 21	2.60	2.00
M.H	+	–	47 ± 9	389 ± 26	0.30	0.10
K.G.	+	–	8 ± 4	311 ± 37	1.75	0.95
		+	17 ± 8	424 ± 16	ND†	ND†
J.F	+	–	19 ± 10	92 ± 11	0.45	0.15
		+	40 ± 9	147 ± 24	0.10	0.25
J.C.	+	–	38 ± 5	210 ± 24	0.50	0.55
H.S.	+	–	4 ± 2	95 ± 22	0.10	0.40
P M.	+	–	19 ± 2	240 ± 42	0.05	0.05
		+	32 ± 20	343 ± 37	0.10	0.20
R.C.	–	–	15 ± 7	65 ± 6	1.35	0.85
		+	22 ± 7	140 ± 21	1.10	0.85
J.D.	–	–	7 ± 1	27 ± 3	1.50	2.25
		+	22 ± 13	190 ± 16	1.50	1.50

DBA, Diamond-Blackfan anemia.

*SEM for three to six determinations

†ND, not done

poietin levels. In addition, the presence of a markedly abnormal erythropoietin dose response in one TEC patient and low CFU-E colony frequency in three TEC patients, supports the observation of Dessypris et al[19] that TEC is a heterogenous disorder which on occasion may mimic DBA.

DISCUSSION

Recent advances in experimental hematology have identified the existence of a pool of erythroid bone marrow progenitors which renew the morphologically identifiable precursor pool.[20-23] The trilineage myeloid progenitor is a cell capable of giving rise to granulocyte/macrophage and megakaryocyte progenitors and to the least mature erythroid progenitor, BFU-E. Under ordinary physiologic conditions in vivo this primitive cell divides and matures to form the next stage of progenitor development, the erythroid colony forming unit or CFU-E. The exact number of erythroid progenitors in human marrow is unknown because they are enumerated only in functional assays. Their differentiation in culture to "normal" numbers of colonies has been thought to indicate the presence of normal numbers of functional progenitors. The development of BFU-E and CFU-E into erythroid colonies has a characteristic pattern of in vitro sensitivities to erythropoietin. The program of differentiation of normal CFU-E is more sensitive to the hormone than is the system that controls the differentiation of BFU-E.[5,20-22]

Failure of erythropoiesis could result from defects at any of the steps in differentiation from progenitor to reticulocyte. Enumeration of progenitor-derived colonies has therefore been used in an attempt to define the level of erythropoiesis that is inhibited in DBA. Nathan et al[6] studied erythroid progenitor-derived colony numbers in a series of patients with DBA, most of whom had had the disease for at least five years and were either transfusion dependent or taking prednisone. None were evaluated at the time of diagnosis. The absence of detectable BFU-E in this group of patients led these investigators to conclude that the defect arises at an early step, the commitment of trilineage progenitors to BFU-E. Freedman and co-workers detected patients with normal as well as low numbers of CFU-E.[7] BFU-E were not measured, and the relationship of the studies to the time of diagnosis was not specified. Variable results have been reported in smaller studies.[8,9,24-26] Nathan et al[6] and Chan et al[8] also demonstrated that the progenitors of some patients with DBA exhibit relative erythropoietin insensitivity. In some of these cases, the erythropoietin dose response could be normalized somewhat by the administration of prednisone in vivo[6] or even in vitro,[8] suggesting to Chan and co-workers[8,9] the possibility that the degree of erythropoietin insensitivity and the response to prednisone in vitro might correlate with a clinical steroid response. The exact mechanism of this in vitro abnormality in progenitor differentiation remains controversial. An intrinsic progenitor defect may be the cause, but other workers have suggested that red cell failure in DBA is caused by the suppression of erythroid differentiation by cytotoxic or autoreactive T lymphocytes[27,28] or by a bone marrow microenvironmental defect.[24] In addition, other abnormalities of lymphocyte function have been observed in this disease.[29] Abnormal lymphocyte function suggests that a suppressor mechanism may be operative. However, transfusion sensitization could explain these results.[30,31] Other investigators have shown that peripheral blood T cells from patients with DBA are able to induce in vitro erythropoietin-dependent BFU-E colony expression as do normal T cells[32] and have no suppressor function.[6,33] One group of investigators appears to have identified a serum factor that inhibits erythropoiesis in DBA,[34] but further attempts to identify such humoral inhibitors have not been successful,[7,24,35] in contrast to the findings in TEC.[18,19]

In this study, erythroid progenitor-derived colonies from patients with DBA were enumerated in cultures containing erythropoietin at a concentration of 2 U/mL. The mean colony frequency observed in DBA was lower than the mean of normal controls and the steroid-resistant patients had values in the lowest range, although the addition of BPA significantly enhanced their CFU-E colony expression. However, if one concludes that a significant frequency of committed progenitors, especially CFU-E, indicates a relatively intact pathway of differentiation from trilineage to committed progenitors, there does not appear to be an in vivo defect in progenitor maturation, especially in early cases of steroid-responsive DBA, that is significant enough solely to account for the observed degree of anemia and reticulocytopenia.

To determine the presence of a functional defect in the erythropoietin-dependent differentiation of DBA progenitors, both colony size and a progenitor erythropoietin dose response was evaluated in each patient studied. Results showed that seven of nine patients had an abnormality in the terminal differentiation of progenitors to colonies of hemoglobinized cells, characterized by these measures of insensitivity to erythropoietin. Although both patients who were refractory to prednisone therapy had abnormal dose responses to crude erythropoietin and small BFU-E–derived colonies, the presence of two responsive patients with identical in vitro abnormalities and three with small BFU-E–derived colonies but a normal erythropoietin dose response indicates that DBA is a heterogenous disorder in which abnormal progenitor differentiation in response to crude erythropoietin using the culture system we use is common. The variation in severity does not necessarily correlate with a lack of clinical responsiveness to prednisone therapy.

The study of bone marrow erythroid progenitors from patients with TEC provides an evaluation of progenitor frequency and differentiation in response to erythropoietin in the face of a level of anemia, reticulocytopenia, and bone marrow erythroid aplasia similar to that seen in DBA. In three of four patients studied, we were able to demonstrate normal BFU-E colony morphology and response to erythropoietin. CFU-E colonies were deficient in three of four TEC patients. The [Epo]½max in these cases was mildly elevated. The defect in two steroid-responsive and in both nonresponsive DBA patients was far in excess of this mild abnormality. We therefore conclude that the normal progenitor frequencies observed in DBA were not due to a concentration artifact and that the abnormal response to erythropoietin in DBA is not merely an epiphenomenon associated with a high degree of erythroid aplasia.

A large body of data suggests that the defect in erythroid differentiation in DBA is a consequence not of an intrinsic progenitor abnormality such as a decreased or abnormal responsiveness to erythropoietin and/or BPA, but of the extrinsic suppression of progenitor differentiation.[24,27,28] Although it is possible that high levels of these mediators may overwhelm a suppressor mechanism, the requirement in severe cases for exogenous BPA to induce the terminal differentiation of DBA CFU-E in vitro and the relative insensitivity of the system to crude erythropoietin also suggest that there could be defective BPA production by DBA marrow accessory cells. The addition of high concentrations of crude erythropoietin may even enhance colony expression in less severe cases by providing exogenous BPA. Thus, the use of impure erythropoietin preparations containing BPA may have obscured an in vitro defect in the progenitor differentiation system in the steroid-responsive patients. Recently, we have been able to fractionate normal marrow into a highly enriched progenitor population and three distinct accessory cell populations.[36] When this system was used, normal CFU-E removed from all sources of BPA have been shown to require small amounts of exogenous BPA to induce erythropoietin-dependent differentiation. The data presented in this study demonstrate that congenital hypoplastic or DBA is a heterogenous disorder. There appears to be no significant block in erythroid maturation from the trilineage myeloid progenitor to CFU-E. However, there is instead a defect in the terminal differentiation system of erythroid progenitors. This observation and further data demonstrating an abnormal response of some DBA progenitors to crude erythropoietin and BPA provide the essential framework for more definitive studies. We should now be able to examine the response of highly enriched progenitors to pure preparations of erythropoietin and BPA and to evaluate bone marrow accessory cell function in DBA. These studies, based on the data presented here, will allow for a more precise understanding of the apparently diverse pathophysiology of DBA.

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Plasma Clearance Rates of Coagulation Factors VIII and IX in Factor-Deficient Individuals

By Dennis A. Noe, William R. Bell, Paul M. Ness, and Jack Levin

The plasma clearance rates of factors IX and VIII were determined in patients with hemophilia A and B who had received factor replacement by prolonged, continuous infusion of factor concentrates. The clearance rates were calculated by dividing the factor infusion rates by the steady-state plasma factor activities corrected for baseline factor activities. The mean factor IX clearance rate in eight factor IX-deficient patients was 233 mL/h (range 159 to 340 mL/h). The mean normalized clearance rate was 3.4 mL/h/kg. The mean factor VIII clearance rate in eight factor VIII-deficient patients was 294 mL/h (range 229 to 361 mL/h) and the mean normalized rate was 5.0 mL/h/kg. Both factors show linear relationships between the factor infusion rates and the steady-state plasma factor activities achieved.

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THE DISAPPEARANCE of procoagulant factors IX and VIII from the plasma of factor-deficient individuals receiving replacement therapy is usually described in terms of first-order elimination rate constants or half-lives.[1] However, both of these kinetic parameters are hybrid measures that depend as much on the volume of distribution of a factor as they do on the rate of elimination of the factor.[2] The plasma clearance rate of a factor, on the other hand, is the primary kinetic parameter that uniquely measures the net removal of the factor from the plasma.[3] In addition, because the plasma clearance rate is a model-independent measure, no assumptions need be made concerning the distribution of a factor in the body or its sites of metabolism or excretion.[4,5] Hence, clearance rate is the logical and preferable way to quantify the disappearance from plasma of the coagulation factors because details concerning distribution, metabolism, and excretion of the factors are unknown.

There are two experimental approaches to the measurement of the plasma clearance rate of a substance.[2] One method is to administer an intravenous (IV) bolus dose of the substances and then to construct its plasma concentration *v* time curve. The clearance rate is calculated by dividing the dose by the area under the curve. In the other method, the substance is infused IV at a constant rate until the steady state is achieved. In this case, the plasma clearance rate equals the infusion rate divided by the steady-state plasma concentration. We have used this relationship to estimate the plasma clearance rates of factors IX and VIII in factor-deficient individuals who received factor replacement therapy in the form of prolonged, continuous infusions of factor concentrates.

MATERIALS AND METHODS

Subjects From 1978 to 1982, many of the factor VIII- and factor IX-deficient patients admitted to The Johns Hopkins Hospital participated in a clinical study in which they received factor replacement therapy using a continuous infusion protocol.[6] This therapeutic approach was used to achieve constant plasma factor levels rather than the varying levels that result from an intermittent dosing regimen. The patients who were treated with this protocol were identified by a search of the medical records maintained by the Division of Hematology. The pertinent demographic, clinical, and laboratory data on these patients were extracted from the individual medical records. Patients with antifactor antibodies were excluded. This study was approved by the Joint Committee on Clinical Investigation of The Johns Hopkins University School of Medicine.

Factor infusion protocol. At least one and sometimes multiple pretreatment blood specimens were collected and assayed for plasma factor activity The patients received two to 16 bolus doses of reconstituted factor concentrate (factor IX concentrate: Konyne, Cutter Laboratories, Berkeley, Calif; factor VIII concentrate: Factorate, Armour Pharmaceutical Company, Kankakee, Ill) on an every-12-hour schedule prior to the start of continuous infusion therapy. The doses of concentrate administered were those believed to be appropriate in the respective clinical situations. The factor content of the concentrate doses were calculated from the manufacturers' stated product content times the volume of concentrate infused. Blood specimens for plasma factor activity determination were drawn ~15 minutes before and 15 to 30 minutes after each dose.

The last of the bolus doses served as a loading dose, immediately after which the continuous infusion of factor concentrate was started. The rate of infusion of concentrate was selected so that the total daily factor activity infused would be equal to that given during the intermittent bolus dose regimen. Each container of infusate was administered over 12 hours; therefore, adequate concentrate to provide one half of the daily total factor activity appropriate for the patient was prepared and placed in the infusion solution The infusion solution was 1 L of 0.2 mol/L of NaCl, 5% dextrose solution, packaged in a polyvinyl plastic bag. The rate and constancy of administration was controlled by a volumetric displacement pump (Abbott/Shaw LifeCare IV Pump, Model II, Abbott Laboratories, North Chicago). Continuous infusion therapy lasted from 60 to 312 hours. Blood specimens were drawn 10 to 30 minutes after the start of each container of infusate and approximately ten minutes prior to the termination of each container. The specimens were obtained by venipuncture of the arm that did not receive the infusion. The infusions were discontinued when indicated clinically. Plasma samples were drawn approximately every six hours after discontinuation of replacement therapy until factor levels of <0.1 U/mL were detected. Factor activities were assayed in all the blood specimens.

Kinetic analysis The plasma clearance rate of the factors was calculated by dividing the factor infusion rate by the corrected steady-state plasma factor activity. plasma clearance rate = infusion rate/steady-state factor activity. Steady-state factor activity was defined as the average of three to five plasma factor activities

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measured once the factor levels had reached an apparent plateau during continuous infusion therapy. The corrected steady-state factor activity equalled the steady-state factor activity minus the baseline factor activity. The average of a patient's pretreatment factor levels constituted the baseline activity.

Analytical methods. Routine procedures for blood collection, sample preparation, and plasma factor assays were used in our patients. Blood was collected in 5-mL samples in plastic tubes containing 0 5 mL of 0.13 mol/L of sodium citrate Within 30 minutes of collection, the samples were centrifuged at 1,800 g for ten minutes, rendering the plasma platelet-poor. The plasma supernates were transferred to plastic tubes and immediately placed into storage at −20°C. Factor assays were performed within 72 hours. Factor VIII and factor IX activities were quantified by one-stage clotting-time bioassays using factor VIII- and factor IX-deficient plasma, respectively, as substrates.[7] The factor deficient plasmas were obtained by plasmapheresis of patients with severe congenital factor deficiencies as determined by family and clinical histories and corroborating laboratory studies. Single clotting-time determinations for sample plasmas were compared with the clotting time *v* plasma concentration dose-response curves for normal, pooled plasma. Sample coagulant activities were interpolated from the control plasma curves as graphed on double logarithmic paper. Normal plasma was obtained from healthy blood bank donors with normal prothrombin and partial thromboplastin times The plasma pools consisted of plasma from 60 to 120 donors. One unit of factor per milliliter was defined as the factor activity in normal human plasma.

RESULTS

Eight factor IX-deficient patients and eight factor VIII-deficient patients were identified as having received factor replacement by continuous infusion of factor concentrate. In every case, replacement therapy was administered either to control a bleeding episode or to provide hemostatic factor levels during and after a surgical procedure. Among the factor IX-deficient patients, 3 had received 1 course of continuous infusion therapy, 3 had 2 courses, 1 had 3 courses, and 1 had 6 courses (Table 1). Among the factor VIII-deficient patients, 6 had received a single course of continuous infusion therapy, 1 had 2 courses, and 1 had 3 courses (Table 2). For 5 of the patients, some or all of the courses of therapy had been conducted during a single hospital stay.

Table 1. Subject Data for Factor IX-Deficient Individuals

	Weight (kg)	Height (cm)	Surface Area (m^2)	Infusion Rate (U/h)	Corrected Steady-State Factor Activity (U/mL)	Clearance Rate (mL/h)
1	61	165	1 65	83	0 36	230
	61			95	0.39	244
2	55	180	1 65	167	0 52	321
3	59	173	1 65	140	0.79	177
4	65.5	178	1.75	51	0.35	146
	59.5			102	0.34	300
5	76	171	1 85	83	0.45	184
	74			102	0.41	249
	75.5			83	0.50	166
	78			81	0.27	300
	78			40	0.22	182
	78			20	0.10	200
6	92	175	2.05	98	0.35	280
	88 5			24	0.09	266
7	75.5	179	1.90	120	0.51	235
	77.5			102	0.30	340
	77.5			51	0.24	213
8	84	174	2.00	51	0.33	159

Clearance rate. Apparent plateaus of plasma factor activity were achieved in all patients after two to five days of therapy. However, three factor IX-deficient patients and three factor VIII-deficient patients exhibited substantial day-to-day variability in their steady-state factor activities (i.e., a range of values ±25% of the mean value). The ranges in these patients, expressed as a percentage of their mean plasma activities, were ±30%, 34%, and 69% for the factor IX-deficient patients, and ±36%, 41%, and 47% for the factor VIII-deficient patients. The factor clearance rate for each patient was calculated by averaging the clearance rates determined from each of the infusions administered to the patient.

The factor IX clearance rates ranged from 159 to 340 mL/h (normalized for body weight: 1.9 to 5.8 mL/h/kg) with a mean value of 233 mL/h (3.4 ml/h/kg). The clearance rates do not correlate with body weight ($r = -.17$) or body surface area[8] ($r = -.13$). The factor VIII clearance rates ranged from 229 to 361 mL/h (3.5 to 5.9 mL/h/kg) with a mean rate of 294 mL/h (5.0 mL/h/kg). The correlations with body weight ($r = .50$) and body surface area ($r = .60$) are not significant.

Dose response. The relationships of the corrected steady-state factor activities to the rates of factor infusion were studied by least-squares linear regression analysis. The results of all of the studies were analyzed. The regression equation for the dose response to factor IX is: corrected steady-state factor IX activity = 0.08 + 0.0034 × factor IX infusion rate (factor IX activity in U/mL, rate in U/h). The regression is statistically significant ($r = .81$, $P < .001$) and, as shown in Fig 1, fits the data well (SE of the estimate = 0.10 U/mL). The analysis of variance of the regression indicates that 65% of the variation in steady-state factor IX activities is attributable to the different infusion rates. Conversely, 35% of the variation is unexplained. Separate regression analysis of the study data from patient 5 indicates that intraindividual variability accounts for >65% of the total unexplained variation. The possibility that variability in body weight accounts for some of the unexplained interindi-

Table 2. Subject Data for Factor VIII-Deficient Individuals

	Weight (kg)	Height (cm)	Surface Area (m^2)	Infusion Rate (U/h)	Corrected Steady-State Factor Activity (U/mL)	Clearance Rate (mL/h)
1	74	175	1 90	106	0.31	342
2	50	165	1 50	50	0.17	294
3	47	162	1.45	101	0.44	229
4	65	175	1.80	150	0.45	333
	74	180	1.95	138	0.57	242
	74			47	0.31	152
5	58	162	1.60	195	0 64	304
6	76	170	1.85	101	0.28	361
7	50	170	1.55	93.3	0.33	283
	50			35	0.15	233
8	56	175	1.65	51	0 16	319

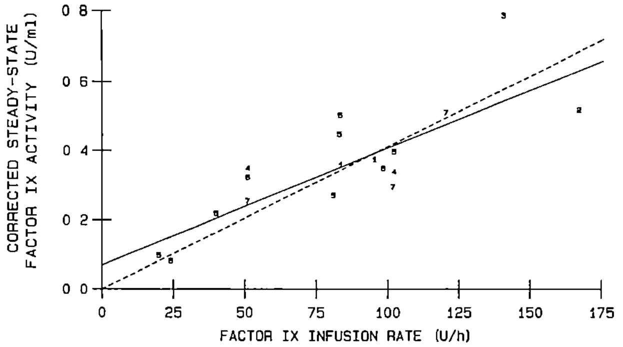

Fig 1. Relationship of the corrected steady-state factor IX plasma activity to the factor IX infusion rate during continuous infusion therapy. The regression line (—) and the regression line passing through the origin (- -) are shown.

vidual variance was tested by regression of the steady-state factor activities on the individual infusion rates divided by the patient's body weight. This approach did not produce a better fit of the data (r = .78; SE of the estimate = 0.10 U/mL).

The regression equation describing the factor VIII dose response is: corrected steady-state factor VIII activity = 0.06 + 0.0030 × factor VIII infusion rate (factor VIII activity in U/mL, rate in U/h). This regression also achieves statistical significance ($r = .91$, $P < .001$) and fits the data well (SE of the estimate = 0.07 U/mL), as shown in Fig 2. Here, the variability in the infusion rates accounts for 76% of the variation in steady-state factor VIII activities. As with the factor IX data, normalizing the factor VIII infusion rates for body weight did not result in an improved fit of the data (r = .87, SE of the estimate = 0.09 U/mL).

Ordinary linear regression analysis generates equations that have nonzero intercepts. The presence of a constant term allows the equations to fit the data better but, as in this case, does not strictly adhere to the form of the physiologic model. Because neither of the constant terms in the ordinary regression lines were significantly different from zero, the data were reexamined by a linear regression analysis in which the fitted lines were forced to pass through the origin. The resulting equations are: corrected steady-state factor IX activity = 0.0042 × factor IX infusion rate (factor IX activity in U/mL, rate in U/h); and corrected steady-state factor VIII activity = 0.0035 × factor VIII infusion rate (factor VIII activity in U/mL, rate in U/h).

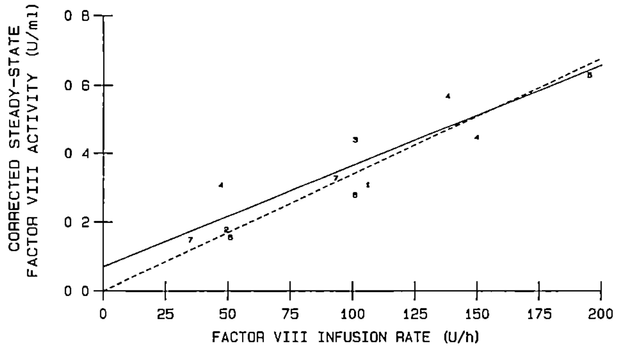

Fig 2. Relationship of the corrected steady-state factor VIII plasma activity to the factor VIII infusion rate during continuous infusion therapy. The regression line (—) and the regression line passing through the origin (- -) are shown.

As shown in Figs 1 and 2, over the range of factor activities achieved in this study, these equations differ very little from those found by ordinary regression analysis. In addition, these equations are essentially identical to those which result from rearrangement of the clearance model equation (first equation) and substitution of the appropriate mean factor clearance rates: corrected steady-state factor IX activity = 0.0043 × factor IX infusion rate (factor IX activity in U/mL, rate in U/h); and corrected steady-state factor VIII activity = 0.0034 × factor VIII infusion rate (factor VIII activity in U/mL, rate in U/h).

The excellent fit of the data confirms the validity of the clearance equation as a mathematical model of factor kinetics during continuous infusion therapy.

DISCUSSION

The reliability of an estimate of the plasma clearance rate of a substance determined with the continuous infusion method depends on accurate measurement of the dose administered and the plasma concentrations achieved. The factor activities in this study were measured by experienced technicians using a standard, quality-controlled, one-stage bioassay.[7] Optimal specimen collection and handling techniques were used and the plasma specimens were stored for <72 hours prior to being assayed. Therefore, there is no reason to suspect any systematic error in the measurement of the factor concentrations.

Because of the retrospective nature of this study, it was necessary to calculate the factor infusion rates by using the manufacturers' stated product content of factor in the concentrates. It would have been preferable to have measurements of the factor contents of the infusates. A number of studies have found that the stated potencies of the high-purity concentrates used in these patients are accurate, however.[9-11] The factor IX concentrate has also been found to contain a minimal amount of activated factor IX.[12] The factor concentrates have also been found to be stable for at least 12 hours when kept at room temperature. This is true for the factor IX concentrate in its reconstituted form, as stated in the product insert (Document 14-7620-203; rev Sept 1978), and for the factor VIII concentrate in dilute solution, the condition under which it was administered in this study.[13]

An additional concern in the measurement of clearance rates using the continuous infusion method is that the plasma concentrations must be measured when the substance is in steady state. If they are measured when the substance is still accumulating in the body, the clearance rate will be underestimated. In this study, the factor activities two to five days after the beginning of infusion therapy were taken to represent the steady-state factor concentrations. During that interval, the activities appeared to have reached a steady-state plateau; ie, the activities were not continuing to increase, although at times there was appreciable day-to-day variability in the measured activities. In addition, by using published descriptions of the bolus kinetics of factor IX[14] and VIII,[1] it can be calculated[15] that factor IX will have reached at least 95% of its steady-state value by 102 hours following the beginning of infusion therapy, and factor VIII will have reached 95% of its steady-state value by 60 hours. Both of these times are within the sampling interval chosen on empirical grounds in this study.

Further evidence that the clearance rate calculations in this study are valid is that the estimates agree with others in the literature. The normalized estimate of the plasma clearance rate of factor VIII, 5.0 mL/h/kg, is close to the rates that can be calculated from two studies of the bolus dose kinetics of factor VIII concentrate. In six patients receiving each of three brands of concentrate, the average clearance rate was 5.5 mL/h/kg.[16] In another 35 patients, each receiving one of three brands of concentrate, the average clearance rate was 3.9 mL/h/kg.[17] The present estimate is also very close to the rate that can be calculated from the data in a report of the dose response of five children to continuous factor infusion therapy using cryoprecipitate and factor concentrate,[13] 5.3 ml/h/kg. The plasma clearance rate of factor IX that can be derived from a study of the bolus dose kinetics of factor IX concentrate in seven patients is 5.0 mL/h/kg.[18] This value compares favorably with the normalized clearance rate in the patients reported here, 3.4 mL/h/kg, although the agreement is not as close as that among the factor VIII clearance rates. In a study of the bolus dose kinetics of radiolabeled factor IX, the clearance rate of factor IX in three patients was found to be 5.3 mL/h/kg (the clearance rate in a fourth subject was much higher, 10.5 mL/h/kg).[19]

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B Lymphoblastoid Cell Lines With Normal and Defective O-Glycosylation Established From an Individual With Blood Group T_n

By Carl G. Gahmberg, Liisa Peltokorpi, and Leif C. Andersson

Individuals with the T_n blood group contain terminal serine/threonine-linked *N*-acetylgalactosamine residues in their blood cells. This is due to lack of UDP-D-galactose: D-*N*-acetyl galactosamine β-D-galactosyl transferase from part of their red cells and probably from their leukocytes. We have established B lymphoblastoid cell lines from such an individual by in vitro infection of his lymphocytes with Epstein-Barr virus. The original line contained a mixture of cells reactive and nonreactive with *Helix pomatia* lectin (Hp). These cells were subcloned after staining with fluorescent Hp by a fluorescence-activated cell sorter (FACS) into homogeneous, phenotypically stable lines of Hp-positive (Hp+) and Hp-negative (Hp−) cells. The molecular differences between the membrane glycoproteins were characterized by carbohydrate-specific surface labeling techniques, Hp affinity chromatography, polyacrylamide slab gel electrophoresis and glycopeptide/oligosaccharide analysis. The major O-glycosidic membrane glycoprotein (GP105) was retained on Hp-Sepharose columns only from Hp+ cells, whereas the common leukocyte antigen (GP160–200) was partially retained on Hp columns from both lines. These proteins were isolated by immune precipitation with monoclonal antibodies and characterized. The results show that the GP105 glycoprotein from Hp+ cells contains terminal *N*-acetylgalactosamine residues but also more complex oligosaccharides. The common leukocyte antigen showed different electrophoretic mobilities in Hp+ and Hp− cells. UDP-galactose D-*N*-acetyl galactosamine β-galactosyl transferase was almost absent in the Hp+ cells. These cell lines are useful for studies on the functional role and regulation of the biosynthesis of O-glycosidic carbohydrates.

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PROTEINS expressed at the surface of mammalian cells are almost always *glyco*proteins.[1] Two major types of carbohydrate structures exist in membrane glycoproteins: asparagine-linked *N*-glycosidic oligosaccharides and serine/threonine-linked O-glycosidic oligosaccharides.[2] In contrast to the relatively large amount of information available on the structure, biosynthesis, and function of *N*-glycosidic carbohydrates, little is known about the biosynthesis and function of O-glycosidic carbohydrates in cell surface glycoproteins.

Structural/functional relationships can be elucidated by use of cellular mutants with impaired glycosylation. Under the selection pressure with various toxic plant lectins or specific antisera against surface molecules, mutant cell clones that lack defined carbohydrate structures have been obtained.[3,4]

Instead of using these methods of selection, another approach would be to immortalize naturally existing cellular mutants. Red cell membranes from individuals with the T_n blood group lack UDP-galactose: *N*-acetyl D-galactosamine β-D-galactosyl transferase.[5,6] Platelets and hematopoietic precursors from T_n-positive individuals are reactive with the *N*-acetyl galactosamine-specific *Helix pomatia* lectin (Hp), indicating a glycosylation change in these cells resulting from a similar enzyme deficiency.[7,8]

We have now established a lymphoblastoid cell line by in vitro Epstein-Barr virus infection of blood lymphocytes from a person with the T_n blood group. Approximately 40% of cells in the original cell line reacted with the Hp lectin. Here we report that the cloned subline consisting of Hp+ cells contains almost no O-galactosyl transferase activity, which results in incomplete O-glycosylation of the major membrane sialoglycoprotein and T_n blood group activity. This cell line should be useful for studies on the function of O-glycosidic carbohydrate.

MATERIALS AND METHODS

Establishment of lymphoblastoid cell lines. Mononuclear leukocytes were isolated by Ficoll-Isopaque density centrifugation from 20 mL of heparinized blood of normal individuals and an individual with the T_n blood group. This individual with blood group O had ∼33% apparently normal red cells and 67% with lower electrophoretic mobility.[9] The isolated blood mononuclear leukocytes were treated for three hours with culture supernatants from the marmoset cell line 95-8, which produces infectious Epstein-Barr virus.[10] The cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Glasgow, Scotland). After approximately one month, lymphoblastoid cells growing in characteristic aggregates were observed.

Isolation of Hp-reactive and nonreactive lymphoblastoid cells. Hp was obtained from Pharmacia (Uppsala, Sweden). An aliquot was labeled with fluorescein using fluorescein isothiocyanate (FITC).[11] The mixture of Hp-reactive (Hp+) and Hp-nonreactive (Hp−) cells was fractionated using FITC-labeled Hp and a FACS IV (Becton Dickinson, Oxnard, Calif). The Hp+ and Hp− cells were further cultured as described above. The cells were studied by fluorescence using labeled Hp and a Zeiss Universal fluorescence microscope.

Cell surface labeling of lymphoblastoid cells. Approximately 50×10^6 cells were labeled with ^3H by treatment with *Vibrio cholerae* neuraminidase (Koch-Light, Haverhill, Suffolk, England) plus galactose oxidase (Sigma, St Louis), or galactose oxidase only, followed by reduction with NaB^3H_4 (8 to 15 Ci/mmol, The Radiochemical Centre, Amersham, England) as described in detail previously.[12] Sialic acid-containing glycoconjugates were labeled by the periodate/NaB^3H_4 surface-labeling technique.[13] After being labeled, the cells were washed in 0.15 mol/L of NaCl to 0.01 mol/L of sodium phosphate, pH 7.4 (PBS) and lysed at 0 °C in PBS containing 1% Triton X-100, 1% ethanol, and 2 mmol/L of phenyl-

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methylsulfonyl fluoride (Sigma). The samples were centrifuged at 10,000 g for 30 minutes and used for subsequent analyses.

Affinity chromatography of radioactively surface-labeled glycoproteins. Periodate/NaB^3H_4-labeled Hp+, Hp− and lymphoblastoid cells from normal individuals were solubilized in Triton X-100-containing buffer, and extracts corresponding to 5×10^6 cells were applied to Hp-Sepharose columns (Pharmacia). After being washed with the Triton X-100-containing buffer, adsorbed glycoconjugates were eluted with this buffer containing 10 mmol/L of D-*N*-acetyl galactosamine. Two-mil fractions were collected, and 25-μL aliquots were counted for radioactivity in a Triton X-114-based scintillation fluid. The passed-through and sugar-eluted peak tubes were separately combined and used for subsequent analysis.

Immunoprecipitation. Immune precipitation was performed as described[14] with some modifications. Surface-labeled cell extracts were preincubated with 0.1 mL of a 10% suspension of protein A-containing *Staphylococcus aureus* cells (Cowan I) for 30 minutes at 0 °C. After centrifugation, the supernatants were recovered and incubated for two hours with the L10 monoclonal antibody[15] or with a monoclonal antibody against the common leukocyte antigen (New England Nuclear, Boston). Then, 5 μL of rabbit anti-mouse IgG was added (Dakopatts, Copenhagen, Denmark), and the incubation was continued for one hour. *S aureus* cells (0.2 mL of the 10% suspension) were added and, after 30 minutes, the bacteria were washed, and the adsorbed antigens were eluted by boiling in 1% sodium dodecyl sulfate (SDS). Aliquots were counted for radioactivity, and the rest were used for gel electrophoresis and glycopeptide/oligosaccharide analysis.

Polyacrylamide slab gel electrophoresis. Polyacrylamide slab gel electrophoresis of radioactively labeled cell extracts was performed according to Laemmli[16] using 8% acrylamide gels. The gels were treated for fluorography according to Bonner and Laskey.[17] ^{14}C-Labeled standard proteins were obtained from the Radiochemical Centre, Amersham. Kodak X-Omat AR film was used.

Analysis of glycopeptides/oligosaccharides. ^3H-Surface-labeled samples from the Hp-Sepharose eluates or immune precipitates were digested with 10 mg/mL of protease from *Streptomyces griseus* (Pronase) (Sigma). The samples were incubated in 0.1% SDS in Dulbecco's PBS for 24 hours at 60 °C. After lyophilization, the alkali-labile oligosaccharides were liberated by treatment with 0.05 *N* of NaOH and 1 mol/L of $NaBH_4$ for 16 hours at 45 °C.[18] After neutralization with acetic acid, the samples were lyophilized and analyzed on a Biogel P-6 column in 0.1 mol/L of NH_4HCO_3 and 0.1% SDS. Blue dextran was added to each run to determine the position of the void volume. Aliquots of the fractions were counted for radioactivity. Standards were: *N*-acetyl neuraminic acid α 2-3 D-galactose β1-3 (*N*-acetyl neuraminic acid α2-6) *N*-acetyl D-galactosaminitol and *N*-acetyl neuraminic acid α2-3 D-galactose β1-3 *N*-acetyl D-galactosaminitol prepared from glycophorin A of human red cells.[19] *N*-acetyl neuraminic acid α2-6 D-*N*-acetyl galactosaminitol was prepared from bovine submaxillary mucin, and ^3H-*N*-acetyl galactosaminitol was made from *N*-acetyl D-galactosamine (Sigma) by reduction with NaB^3H_4.

Assay of UDP-D-galactose: N-*acetyl D-galactosamine β-D-galactosyl transferase activity.* Assay for galactosyl transferase activity was essentially performed according to Cartron et al.[5] Lymphoblastoid cell samples containing 1 mg of protein were solubilized in 130 μL 1% Triton X-100 in PBS at 0 °C. After centrifugation at 10,000 g for ten minutes, the supernatants were recovered. The assay mixture contained 80 μL of cell extract, 40 μL of 0.1 mol/L sodium cacodylate buffer pH 7.0, 20 μL of 0.1 mol/L $MnCl_2\,4H_2O$, 33 μL of 15.1 mmol/L naphtol α-*N*-acetyl D-galactosamine (Koch-Light), 0.25 μCi of UDP-^{14}C-D-galactose (Amersham), and 10 μL of 5% Triton X-100. The samples were incubated at 37 °C, and aliquots were removed at times indicated. The analysis was performed by means of descending chromatography in ethylacetate–pyridine–H_2O (10:4:3) on Whatman 1-mm paper for 12 hours. After being dried, the paper strips were cut into 1-cm pieces and counted for radioactivity in the Triton X-114-band scintillation fluid. The $Rf_{glucose}$ of galactose β1-3 *N*-acetylgalactosamine-α-*p*-nitrophenol was 2:1.

RESULTS

Establishment and general characteristics of T_n-positive and T_n-negative lymphoblastoid cell lines. Epstein-Barr virus infection of blood mononuclear cells results in polyclonal activation and immortalization of B lymphocytes. The lymphoblastoid cell line established from the T_n-positive individual contained ~40% of cells reactive with Hp. The Hp+ and Hp− cells were cloned into sublines by use of the FACS IV after being stained with FITC-conjugated Hp. The Hp+ and Hp− sublines have retained their original phenotypes as regards to lectin reactivities after cultivation for more than one year. Figure 1A shows Hp fluorescence of the unfractionated cells, Fig 1C shows the Hp− cells and Fig 1E

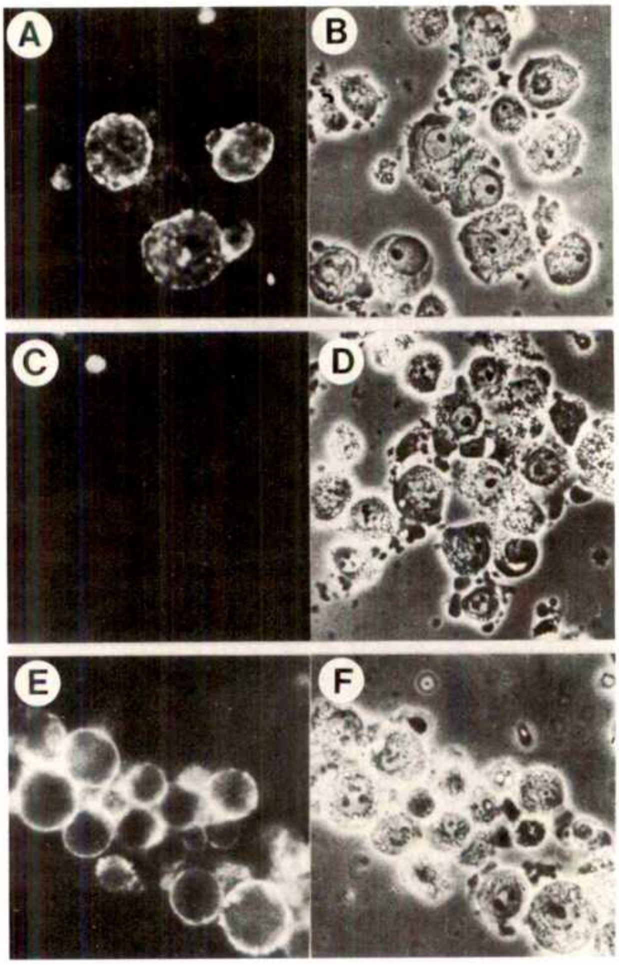


Fig 1. Visualization of *Helix pomatia* lectin positive and negative (Hp+ and Hp−) lymphoblastoid cells by immunofluorescence. The cells to the left are stained with fluorescent Hp, whereas those to the right are visualized by phase contrast illumination. (A and B) Unfractionated lymphoblastoid cells from the T_n-positive individual; (C and D) Hp− cell line; (E and F) Hp+ cell line.

shows the Hp+ cells. Fluorescence was seen only in the Hp+ subline. Both the Hp+ and Hp− cells grow in aggregates characteristic of B lymphoblastoid cells. No differences have been observed in their growth characteristics.

Polyacrylamide gel electrophoresis patterns of galactose oxidase/NaB^{3}H$_4$-labeled cells. Figure 2 shows a polyacrylamide gel of surface-labeled Hp+ and Hp− cells. After treatment with neuraminidase plus galactose oxidase followed by reduction with NaB^{3}H$_4$, the patterns of both cell lines looked similar (Fig 2 B and D). The GP160 through GP200 bands represent the common leukocyte antigen; the GP120 band represents the major O-glycosidic sialoglycoprotein. If the cells were oxidized with galactose oxidase without prior neuraminidase treatment, the patterns were distinctly different. Both the Hp+ and Hp− cells contained label in the common leukocyte antigen bands and in a GP250 glycoprotein, but the GP105 protein was strongly labeled only in Hp+ cells (Fig 2C).

Affinity chromatography of periodate/NaB^{3}H$_4$-labeled cell glycoproteins and their characterization. Detergent-soluble extracts from Hp+, Hp−, and normal periodate/NaB^{3}H$_4$-labeled cells were analyzed on Hp-Sepharose columns (Fig 3). Substantially more radioactivity was retained and eluted from Hp+ cells (Fig 3A), than from Hp− (Fig 3B) or normal lymphoblastoid cells (Fig 3C). Polyacrylamide gel electrophoretic analysis of the Hp+ and Hp− cell extracts chromatographed on Hp-Sepharose columns showed that the GP105 and a diffuse band just below it were the major glycoproteins eluted from Hp+ cells (Fig 4C). The GP160 band was also enriched in the eluate from Hp+ cells, whereas the GP250 glycoprotein was recovered from both cell extracts (Fig 4C and F).

The sugar-eluted peaks from the Hp-affinity chromatographies were subjected to Pronase and alkaline borohydride treatments to liberate glycopeptides/oligosaccharides. The labeled samples were then analyzed by gel filtration on Biogel P-6 columns (Fig 5).

The Hp+ cell glycoproteins contained both *N*-glycosidic and O-glycosidic oligosaccharides (Fig 5A). The complex-type of *N*-glycosidic oligosaccharides elute near the void volume (peak 1). Peak 2 contains O-glycosidic oligosaccharides, which elute in a position similar to that of the major O-glycosidic tetrasaccharide of glycophorin A from red cells.[20] Peak 3 corresponds to the position of *N*-acetyl neuraminic acid α 2–6 *N*-acetyl D-galactosaminitol. When the samples were treated with Pronase only, all radioactivity eluted at the void volume. As was expected, the Hp− and the normal lymphoblastoid cell extracts contained only small amounts of Hp-bound and sugar-eluted glycopeptide/oligosaccharide material (Fig 5 B and C).

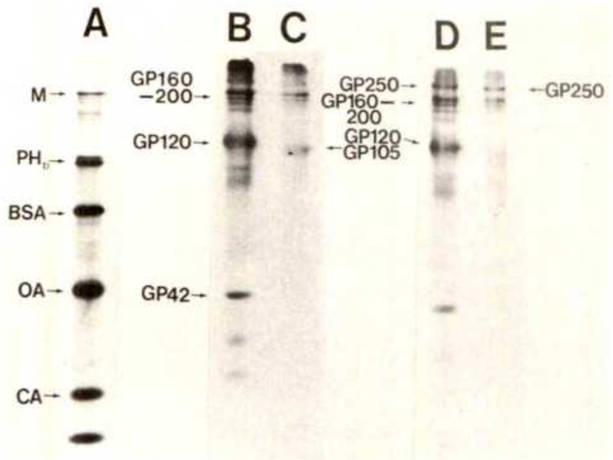
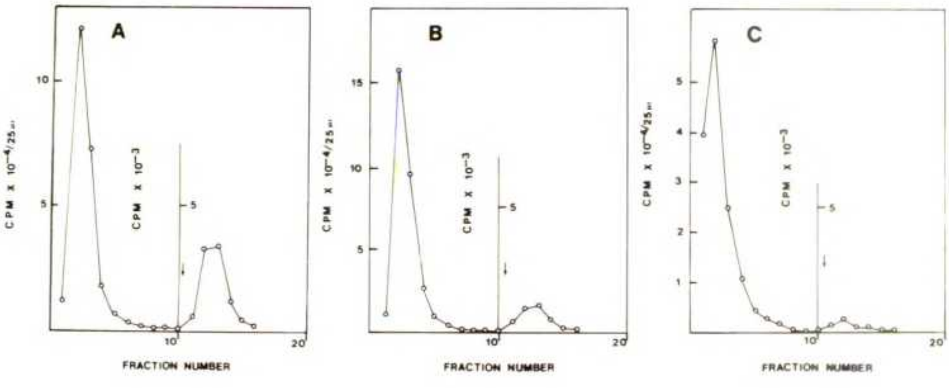


Fig 2. Polyacrylamide slab gel electrophoresis pattern of galactose oxidase/NaB^{3}H$_4$-labeled cells. (A) pattern of ^{14}C-labeled standard proteins. M, myosin; PH$_b$, phosphorylase b; BSA, bovine serum albumin; OA, ovalbumin; CA, carbonic anhydrase. (B) Surface glycoproteins of *Helix pomatia* lectin-positive (Hp+) cells labeled by treatment with neuraminidase and galactose oxidase/NaB^{3}H$_4$. GP160 through GP200, surface glycoproteins with apparent mol wt of 160,000 to 200,000, etc. (C) Surface glycoproteins of Hp+ cells labeled by galactose oxidase/NaB^{3}H$_4$. (D) Surface glycoproteins of Hp− cells labeled by treatment with neuraminidase + galactose oxidase/NaB^{3}H$_4$. (E) Surface glycoproteins of Hp− cells labeled by galactose oxidase/NaB^{3}H$_4$.

Immune precipitation and analysis of the major radioactively surface-labeled glycoproteins. Cell surface labeling by galactose oxidase/NaB^{3}H$_4$ showed label mainly in the GP160–200 and GP105 proteins in Hp+ cells. These proteins were isolated by immune precipitation (Fig 6). The L10 antibody reacted with the labeled GP105 protein from Hp+ cells (Fig 6B), but only a weakly labeled diffuse band with higher apparent mol wt was seen in Hp− cells (Fig 6E). The monoclonal antibody against the common leukocyte antigen reacted well with labeled proteins from both Hp+ and Hp− cells. The precipitated glycoproteins from Hp+ cells had apparent mol wt of 180,000 to 200,000 (Fig 6C), whereas

Fig 3. Affinity chromatography patterns of periodate/NaB^{3}H$_4$-labeled lymphoblastoid cell surface glycoproteins. (A) Pattern obtained with Hp+ cells. (B) Pattern obtained with Hp− cells. (C) Pattern obtained with lymphoblastoid cells originating from a normal individual. Aliquots were counted for radioactivity (25 µl/2 mL). Arrows mark the addition of N-acetyl galactosamine-containing buffer.



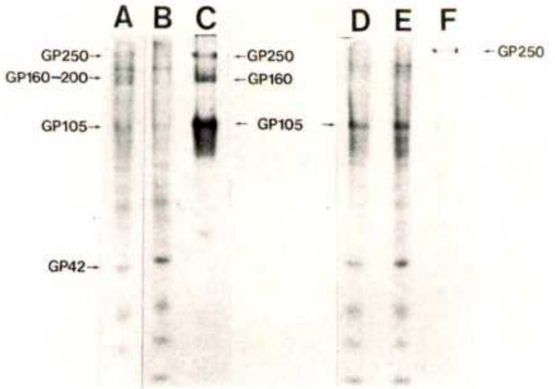


Fig 4. Polyacrylamide slab gel electrophoresis patterns of *Helix pomatia* lectin (Hp)-Sepharose unbound and sugar-eluted periodate/NaB^{3}H$_4$-labeled glycoproteins. (A) Pattern of periodate/NaB^{3}H$_4$-labeled Hp+ cells. (B) Pattern obtained of the unbound glycoproteins from Hp+ cells. (C) Pattern obtained of the lectin-bound and eluted glycoproteins from Hp+ cells. (D) Pattern of periodate/NaB^{3}H$_4$-labeled Hp− cells. (E) Pattern obtained of the unbound glycoproteins from Hp− cells. (F) Pattern obtained of the lectin-bound and eluted glycoproteins from Hp− cells. Apparent mol wt of some major glycoproteins is indicated.

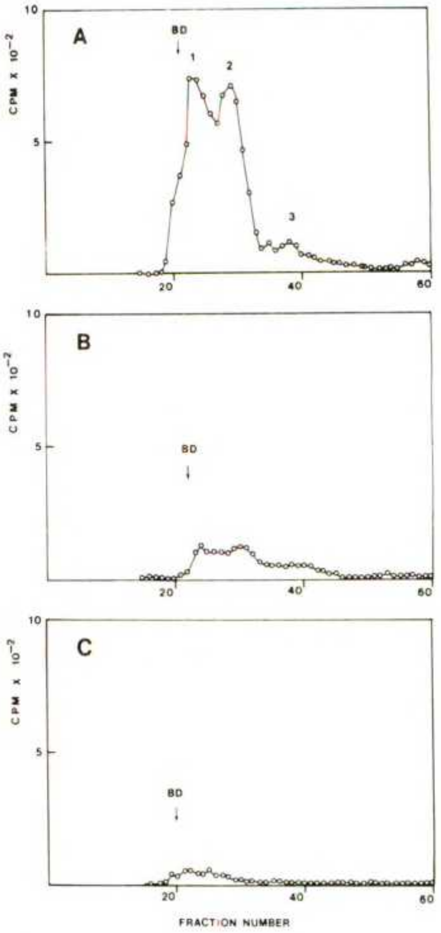


Fig 5. Biogel P-6 gel filtration patterns of *Helix pomatia* lectin (Hp)-Sepharose sugar-eluted fractions treated with Pronase and alkaline borohydride. Similar amounts of periodate/NaB^{3}H$_4$-labeled cells were used for the experiments. (A) Pattern obtained with Hp+ cells. (B) Pattern obtained with Hp− cells. (C) Pattern obtained with lymphoblastoid cells originating from a normal individual. BD, position of Blue dextran. Peak 1 corresponds to the elution volume of *N*-glycosidic complex-type glycopeptides, peak 2 corresponds to the elution position of *O*-glycosidic tetrasaccharides, and peak 3 corresponds to *N*-acetyl neuraminic acid 2-6 *N*-acetylgalactosaminitol.

that from Hp− cells had an apparent mol wt of 160,000 (Fig 6F).

The galactose oxidase/NaB^{3}H$_4$-labeled glycoproteins obtained with the L10 antibody were digested with Pronase and subjected to alkaline borohydride treatment. Gel filtration showed one major peak from the Hp+ GP105 protein, which eluted in the position of N-acetyl galactosaminitol. No peak was obtained from Hp− cells.

The GP105 proteins were also isolated from periodate/NaB^{3}H$_4$-labeled Hp+ and Hp− cells by immune precipitation with the L10 antibody. Biogel P-6 gel filtration showed the presence of complex N- and O-glycosidic glycopeptides/oligosaccharides in both Hp+ cells (results not shown).

UDP-D-galactose N-*acetyl D-galactosamine β-D-galactosyl transferase activities in Hp− and Hp+ cells.* The Hp− cells showed high O-glycosidic galactosyl transferase activity, whereas the activity in Hp+ cells was only 10% to 12% of that (Fig 7).

DISCUSSION

A major unsolved problem in cell biology is the general function(s) of cell surface carbohydrate.[21] The large amount of sugar transferases and processing enzymes needed to build up the diverse carbohydrate chains point to essential roles for these structures. Oligosaccharides are already known to be important in some cases of intercellular adhesion,[22,23] ligand binding,[24,25] in the intracellular transport of newly synthesized glycoproteins,[26,27] and in sorting of lysosomal enzymes.[28-30]

One way of studying functions of carbohydrate groups is to inhibit their synthesis and addition to polypeptides. Several compounds are known to interfere with the synthesis of *N*-glycosidic oligosaccharides. These include tunicamycin, which inhibits the formation of the lipid intermediates,[31] and the different agents which affect the processing of the N-glycosidic oligosaccharides.[32] In some cases, the alterations of cell surface carbohydrates markedly affect the biosynthesis, location, and function of the glycoproteins. No inhibitors or modifiers of the synthesis of O-glycosidic oligosaccharides have been found, however.

Another approach is to use cellular mutants defective in glycosylation. Several such cell lines are available,[3,33] and they have been useful for the elucidation of *N*-glycosidic oligosaccharide processing,[34] and of intracellular glycoprotein transfer routes.[35,36] All described mutants with one exception are specifically affected in *N*-glycosylation. Using the *Vicia villosa* lectin, Conzelmann and Kornfeld recently described a murine cytotoxic T cell mutant lacking terminal β-D-*N*-acetyl galactosamine residues in O-glycosidic oligosac-

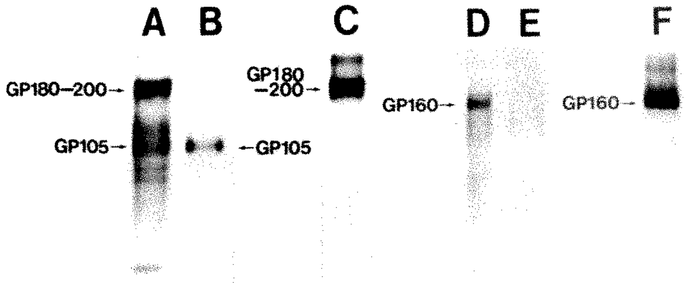


Fig 6. Polyacrylamide gel electrophoresis patterns of immunoprecipitates from *Helix pomatia* lectin positive and negative (Hp+ and Hp−) cells labeled by the galactose oxidase/NaB^{3}H$_4$ technique. (A) Pattern of ^{3}H-labeled Hp+ cells. (B) Pattern obtained with L10 monoclonal antibody from Hp+ cells. (C) Pattern obtained with monoclonal antibody against common leukocyte antigen from Hp+ cells. (D) Pattern of ^{3}H-labeled Hp− cells. (E) Pattern obtained with L10 antibody from Hp− cells. (F) Pattern obtained with monoclonal antibody against common leukocyte antigen from Hp− cells.

charides,[37] which is due to absence of UDP-*N*-acetylgalactosamine: galactose 1,4-*N*-acetylgalactosaminyl transferase.[38] This oligosaccharide structure is evidently uncommon and is probably located on the major sialoglycoprotein of cytotoxic murine T lymphocytes.[39,40]

The receptor for low-density lipoprotein is well characterized. It is highly O-glycosylated.[41] Different functionally defective mutants of this receptor have been described, some of which may be caused by altered O-glycosylation.[42]

Red blood cells of the T$_n$ blood group are polyagglutinable by normal human sera, due to the exposure of terminal *N*-acetyl galactosamine residues in a certain proportion of the red cells which react with naturally occurring antibodies. The molecular defect is known to be due to a decrease or absence of UDP-D-galactose: *N*-acetyl D-galactosamine β-D galactosyl transferase,[5,6] a key enzyme in the biosynthesis of O-glycosidic oligosaccharides.

A comparison between the Hp+ and Hp− lymphoblastoid cells described here shows that the Hp+ cells had only 10% to 12% of the O-glycosidic galactosyl transferase activity of the Hp− cells. This activity may be due to some contaminating Hp− cells. The labeling studies of Hp+ cells using neuraminidase-galactose oxidase/NaB^{3}H$_4$ or periodate/NaB^{3}H$_4$ clearly showed that several surface glycoproteins contained penultimate galactosyl/*N*-acetyl galactosaminyl residues and sialic acids, indicating that there is no large change in *N*-glycosylation.

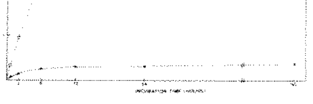


Fig 7. UDP-galactose: *N*-acetylgalactosamine β-galactosyl transferase activity in *Helix pomatia* lectin positive and negative (Hp+ and HP−) cells. The galactosyl transferase activities were measured as described in the Materials and Methods section. The samples were incubated at 37 °C for the times indicated; (O——O) Hp− cells; (x——x) Hp+ cells.

The Hp+ cells contained terminal *N*-acetyl galactosamine residues in the major surface-labeled sialoglycoprotein GP105, which was responsible for the binding to Hp. This sialoglycoprotein and its other leukocyte counterparts[43] are in some respects similar to the major O-glycosidic sialoglycoprotein of red cells, glycophorin A. When glycophorin A was treated with neuraminidase under nondenaturing conditions, its apparent mol wt increased, due to removal of peripheral galactose-linked sialic acids.[19] Similarly, the apparent mol wt of the major leukocyte sialoglycoprotein decreased after neuraminidase treatment (Fig 2).[44,45] The GP105 glycoprotein is probably functionally important. Its T lymphocyte equivalent GP115, reactive with the L10 monoclonal antibody, is either reduced in quantity or absent from lymphocytes of patients with the Wiscott-Aldrich syndrome.[15] These patients suffer from severe immune deficiency.

The GP105 glycoprotein contained O-glycosidic oligosaccharides both in Hp+ and Hp− cells. Although the structure of the larger oligosaccharides is not known, some of them may contain the core structure *N*-acetyl glucosamine β 1–3 *N*-acetyl galactosamine, which has been found in O-glycosidic oligosaccharides.[46,47] In this way, the biosynthesis block caused by the absence of galactosyl transferase could be bypassed.

The common leukocyte antigen is a surface glycoprotein complex showing differing apparent mol wt in T and B lymphocytes.[48-51] These differences have been presumed to be due to varying degrees of glycosylation, but differences in the polypeptide portions have not been ruled out. The observed differences between the common leukocyte antigens of Hp+ and Hp− cells must be due to differences in glycosylation, resulting in changes in apparent mol wt.

It is obviously important to have both "normal" and T$_n$-positive cell lines from the same individual. The cells are useful for studies on the role of O-glycosidic oligosaccharides in the biosynthesis of the GP105 protein and possibly of viral glycoproteins containing O-glycosidic carbohydrate that use the host cell systems for their glycosylation.[52] Until now, few studies have been done on the biosynthesis and O-glycosylation of membrane proteins. Only glycophorin A has been studied in some detail, and its biosynthesis has already revealed a remarkably complex series of events.[53]

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In Vivo Induction of Terminal Differentiation of Malignant Myelopoietic Progenitor Cells by CSF-Inducing Biological Response Modifiers

By Erich Schlick and Francis W. Ruscetti

We have investigated the mechanisms by which colony-stimulating factor (CSF)–inducing biological response modifiers (BRM) may have beneficial effects on tumor-bearing hosts undergoing anti-tumor therapy. First, we have documented that treatment of mice with the chemically defined BRM maleic anhydride divinyl ether copolymer (MVE-2), which induces CSF secretion by macrophages (Mϕ) and bone marrow cells (BMC), significantly increased growth and differentiation of normal myelopoietic cells and counteracted the myelosuppressive effects of cyclophosphamide (CY). Second, we established that MVE-2 may exert CSF-mediated antitumor effects on certain leukemic tumor cells. Serum from mice pretreated in vivo with MVE-2, which contained CSF, induced terminal differentiation of cloned tumor cells from the CSF responsive WEHI-3B D$^+$ subline in vitro, but not from the WEHI-3B D$^-$ subline, which is unresponsive to CSF. In vivo experiments showed that treatment of mice bearing the WEHI-3B D$^+$ tumor first with CY and three days later with the CSF inducer MVE-2, significantly increased their survival time and rendered 20% to 50% of the tumor-bearing mice disease free. No such effects were obtained in mice bearing the WEHI-3B D$^-$ tumor. Thus, the induction of CSF or other differentiation factors by some BRMs may result in thereapeutic effects against certain leukemias based on at least two distinct mechanisms: In addition to their restorative effects on normal bone marrow functions, CSF-inducing BRMs may also prevent further leukemogenesis by induction of terminal differentiation of leukemic cells.

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SEVERAL reports indicate that some immunotherapeutic agents can stimulate in vivo normal myelopoiesis, presumably mediated by CSFs,[1,2] in addition to having an immunomodulating effect on Mϕ and natural killer (NK)–cell activities.[3,4] Since major treatment modalities for various cancers include chemotherapy and/or radiotherapy, which often result in severe suppression of hematopoiesis and immune functions,[5,6] treatment with selected biologic response modifiers (BRMs) might be advantageous to the tumor-bearing host undergoing cytoreductive tumor therapy, (1) by augmenting the host's natural immunity (Mϕ and/or NK cytotoxicity) against tumor cells, (2) by restoring the host's suppressed hematopoietic functions, and (3) possibly by inducing terminal differentiation of certain leukemic tumor cells.

Studies of several murine myeloid leukemia cell lines, especially of the WEHI-3B myelomonocytic leukemia, have shown that these undifferentiated tumor cells could be induced in vitro to differentiate into macrophages (Mϕ) and/or granulocytes, which then exhibit properties similar to those of their normal counterparts.[7,8] Exposure to a variety of chemical agents is able to induce such differentiation in these myeloid leukemia cell lines,[9,10] but analogous differentiation could also be induced by biologic materials, such as postendotoxin serum[11,12] and certain conditioned media.[12,13] Biochemical analysis of these biologic materials indicate that physiologic regulatory molecules such as colony-stimulating factors (CSF), which control in vitro growth and differentiation of normal granulocytes and/or Mϕ, can induce terminal differentiation of myelomonocytic leukemias.[8,12] Of special interest have been the findings that only two out of three different subclasses of CSF are able to induce terminal differentiation. G-CSF, controlling normal granulocyte formation, exhibited high differentiation-inducing activity.[7,13] GM-CSF, controlling normal granulocyte and Mϕ formation, was found to have only weak differentiation-inducing activity, whereas M-CSF, controlling normal Mϕ formation, had no detectable effect on differentiation.[8,14] Since previous studies have indicated that MVE-2 (maleic anhydride divinyl ether copolymer), a chemically defined BRM, might induce increased in vivo secretion of CSFs by Mϕ and BMC[2], in addition to activating Mϕ and NK-cell cytotoxicities[3], we were therefore interested in testing the ability of MVE-2 to modulate (1) the host's myeloid progenitor and immune functions in vivo before and after antineoplastic treatment with cyclophosphamide (CY) and (2) to define the potential of MVE-2 for differentiation of WEHI-3B leukemic cells.

MATERIALS AND METHODS

Male BALB/c mice were obtained from the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, NCI-FCRF, NIH, Frederick, Md The animals were 6 to 8 weeks of age before they were used for experiments.

Maleic anhydride divinyl ether copolymer (MVE-2) was the generous gift of Dr R. Corrano (Adria Laboratories, Columbus, OH). Lipopolysaccharide from *S. typhimurium* (LPS) was purchased from Difco Laboratories (Detroit, Mich). The alkylating agent cyclophosphamide (CY) was obtained from Sigma Chemical (St. Louis) The media and BRM preparations were tested for endotoxin contamination with the Limulus lysate assay and found to contain less than 0.125 ng/mL.

Tumor cells. WEHI-3B leukemia cells were originally obtained from mineral oil–injected BALB/c mice in 1968 [15] This leukemic cell line grows in liquid culture as a population of undifferentiated blast cells and promyelocytes. The leukemic cells form large colonies in agar medium that are composed of similar cells. Their plating efficiency varies from 30% to 70% [14] The WEHI-3B D$^+$ and WEHI-3B D$^-$ sublines were a generous gift from Dr Peter Ralph, Sloan-Kettering Institute, New York Cells of the WEHL-3B D$^+$ subline are responsive to induction of differentiation, whereas cells of the WEHI-3B D$^-$ subline are unresponsive to the induction of differentiation.[16,17] The present studies were performed using

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WEHI-3B D$^+$ and D$^-$ cells grown in liquid suspension culture in RPMI medium with 10% FCS and containing 0.5–2.0 × 10^6 cells/mL. Both cell lines have a doubling time of about 20 hours in vitro and of about 24 hours in vivo. WEHI-3B D$^+$ and D$^-$ cells are equally sensitive to the in vitro cytoreductive effects of an active metabolite of CY, 4-sulfoethylthiocyclophosphamide [4-SET-CY; Asta Z7557][18,19] (donated by Dr P. Hilgard from Asta Werke, Bielefeld, West Germany). The respective ED_{50} (concentration inducing 50% of the maximal effect) for 4-SET-CY-induced growth inhibition were 3 9 μg/mL (WEHI-3B D$^-$ cells) and 5.0 μg/mL (WEHI-3B D$^+$ cells) for a 1 hour incubation with 4-SET-CY (data not shown).

Agar cultures of WEHI-3B D$^+$ and D$^-$ cells were prepared using cloned cells grown in suspension culture in complete RPMI medium [RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY), 292 μg/mL L-glutamine (Gibco) and 100 μg/mL gentamycin (Sigma)]. All semisolid agar cultures were prepared as 1 mL cultures in 35 mm Petri dishes using an equal volume mixture of double strength Dulbecco's modified Eagle's medium and 0.6% agar, supplemented with 20% FCS, 292 μg/mL L-glutamine (Gibco), and 100 μg/mL gentamycin (Sigma). The WEHI-3B cells were added to the liquid agar medium and 1 mL volumes containing 300 cells were pipetted into each culture dish containing 0.1 mL of serially diluted serum from mice pretreated 3 hours before with 5 μg/mouse of LPS (postendotoxin serum)[11,12] or 0.1 mL of serially diluted sera from MVE-2–treated mice Serially diluted conditioned media (CM) from macrophages (Mϕ) or bone marrow cells (BMC) from MVE-2–treated mice were tested likewise. The cultures were allowed to gel and then incubated for 7 days at 37 °C and 8% CO_2 in a fully humidified atmosphere. Colonies (>50 cells) were counted after 7 days of incubation and were classified after Wright staining in situ as undifferentiated if they consisted of a tight aggregate of cells with no outlying cells.[7,14] Differentiated colonies were composed of loosely dispersed differentiating granulocytes/Mϕ around a central aggregate.[7,14] Cells were terminally differentiated, when they failed to form new tumor colonies after being replated in subsequent agar cultures without addition of growth and/or differentiation factors.

Macrophages. Resident peritoneal macrophages (Mϕ) harvested by peritoneal lavage, were seeded into 16 mm wells (Costar, Cambridge, Mass) in 1 mL of complete medium. The cultures were incubated for 2 hours and the adherent Mϕ-monolayers (>95% Mϕ as determined by esterase and Wright staining) were washed twice before testing in experiments. The assay for measuring the macrophage-mediated cytotoxicity has been previously described.[20] Briefly, to measure in vivo activation, monolayers of 8 × 10^5 peritoneal Mϕ, harvested at different times after in vivo treatment of the mice with MVE-2, were overlaid with 8 × 10^4 MBL-2 tumor cells in complete medium and 10 ng/mL of LPS were added. All cultures were incubated for an additional 48 hours at 37 °C and 5% CO_2 and viable MBL-2 cells were then determined by trypan blue dye exclusion. The percent inhibition of MBL-2 cell growth due to Mϕ activation was calculated by:

$$\%\ \text{inhibition} = 1 - \frac{\text{mean of MBL cells from drug treated M}\phi}{\text{mean of MBL-2 cells from nontreated M}\phi} \times 100$$

Bone marrow cells. Sterile single-cell suspensions from femoral bone marrow were prepared as previously described.[21] The number of viable nucleated cells in the femur of each mouse was counted in a hemocytometer. To determine the number of granulocyte-Mϕ committed stem cells (GM-CFU-C), a previously described in vitro soft agar culture technique was used.[22] Briefly, 10^5 unfractionated bone marrow cells (BMC) were suspended in 2 mL of 0.3% agar, supplemented with 15% horse serum (Gibco), 15% fetal calf serum (Gibco), and 100 μg/mL of gentamycin/mL (Sigma), and were plated in 35 mm Petri dishes (Falcon, Oxnard, Calif). As CSF source, 0.2 mL supernatant from WEHI-3B cells[17] was used. The cultures were incubated at 37 °C in a fully humidified atmosphere of 7% CO_2 in air. Colonies (>40 cells) were scored and classified on the basis of their morphology at days 7 and 10 of the culture period.

CSF assay. Cell-free supernatants from in vitro- or in vivo-activated Mϕ and mononuclear BMC were harvested after 48 hours of subsequent in vitro culture (37 °C and 5% CO_2) at a cell density of 10^6 Mϕ/mL or 10^7 BMC/mL and kept at −20 °C until assayed CSF concentrations were then determined using a bioassay as previously described.[22,23] Briefly, serial dilutions of the supernatants or sera to be tested for CSF were added to each 35 mm Petri dish containing 10^5 nonadherent BMC from normal mice in 2 mL of 0.3% agar, supplemented with 15% horse serum, 15% fetal calf serum, and 100 μg/mL gentamycin. At the end of the culture period (10 days at 37 °C and 7% CO_2), the colonies (>40 cells) were counted and classified on the basis of their morphology. No colony formation was noted in the absence of either the standard CSF (supernatant from the WEHI-3B cell line), a supernatant from Mϕ or bone marrow cells, or serum. CSF activity is expressed as U/mL.[23,24] The Student's *t* test was used for statistical analysis.

RESULTS

The results of the experiments on the effects of in vivo treatment with MVE-2 on CSF secretion and myeloid progenitor cells in normal and CY-pretreated mice are summarized in Table 1. MVE-2 at a dose of 25 mg/kg induced an increase in CSF secretion by BMC and Mϕ and in serum CSF levels which was followed by significant augmentation in granulocyte-Mϕ progenitor cells and in nucleated BMC per femur (Table 1, group 2 *v* 1 and Fig 1). Optimal effects could be found 3 days after treatment with MVE-2 at 25 mg/kg and lower or higher doses of MVE-2 were less effective.[2] MVE-2, when given 3 days after the bone marrow suppressive agent CY (150 mg/kg),[5] also induced increased secretion of CSF by Mϕ and BMC, stimulated growth and differentiation of myelomonocytic progenitor cells, and thus restored CY-caused myelosuppression (Table 1, group 6 *v* 5). MVE-2, given 1 day after CY, however, failed to enhance CSF secretion by either Mϕ or BMC, or to counteract the myelosuppressive effects of CY (Table 1, group 4 *v* 3).

Sera from MVE-2–treated mice induce differentiation in WEHI-3B D$^+$ leukemia cells. Next, we investigated whether MVE-2–induced CSF would cause in vitro differentiation of WEHI-3B D$^+$ cells, as compared to WEHI-3B D$^-$ cells. Incubation of WEHI-3B D$^+$ cells in agar cultures with sera derived from MVE-2–treated mice (14 hours after injection) caused a significant increase in differentiation into granulocytes/Mϕ of the leukemia cells (up to a serum dilution of 1/64) as compared to sera from PBS-treated animals (Table 2). The differentiation induced in these WEHI-3B D$^+$ cells was in about two thirds of the cases terminal as shown by the failure of these differentiated cells to form new tumor colonies when replated in subsequent agar cultures without any growth and/or differentiation factors (data not shown). Sera from MVE-2–treated mice (harvested 14 hours after injection) also significantly inhibited (up to a serum dilution of 1/32) the growth of WEHI-3B D$^+$ cells in liquid suspension cultures (Fig 2). The effects of sera from mice harvested at different times after treatment with

Table 1. Effect of MVE-2 on CSF Secretion and Bone Marrow Cellularity in Normal and Cyclophosphamide-Pretreated Mice

Group *	Cyclophosphamide (150 mg/kg IP) Treatment at	Treatment at Day 3 With MVE-2 (25 mg/kg IP)	Determination of GM-CFU-C/Femur (% of Control)	BMC/Femur (× 10^6)	CSF Secretion† U/10^6 Mφ	CSF Secretion† U/10^7 BMC
1	—	–	100	9.9	70	35
2	—	+	142‡	13.7‡	165‡	87‡
3	d 4	–	26‡	3.6‡	81	25
4	d 4	+	42‡	4.4‡	90	38
5	d 6	–	63‡	7.3‡	108	42
6	d 6	+	114‖	11.9‖	198‖	96‖

*BALB/c mice were pretreated with CY at the days indicated. They received additionally a single injection of PBS or MVE-2 at day 3. The mice were killed at day 0 for determination of bone marrow cellularity and CSF secretion by Mφ and BMC. The results shown here are the mean of a typical experiment (5 mice per group), SD was less than 15% of the mean. The experiment was repeated 2 times with comparable results.

†CSF secretion by in vivo-activated Mφ and BMC was determined after 2 days of subsequent in vitro culture of 10^6 Mφ/mL or 10^7 BMC/mL in complete medium without any stimulus

‡$P < 0.01$ as compared to group 1

‖$P < 0.01$ as compared to group 5

MVE-2 on terminal differentiation of WEHI-3B D$^+$ cells paralleled their stimulatory effects on normal myelopoiesis at all times (Fig 1). Incubation of WEHI-3B D$^+$ cells in agar or liquid suspension cultures with serial dilutions of conditioned media from Mφ and BMC of MVE-2–treated mice gave similar results with respect to terminal differentiation (Table 3) and growth inhibition (data not shown) as sera from the same animals. The effects of CM from Mφ or BMC harvested at different times after treatment with MVE-2 on terminal differentiation of WEHI-3B D$^+$ cells paralleled their stimulatory effects on normal myelopoiesis at all times (data not shown). Postendotoxin serum served as a positive internal control.[11,12] Cells of the WEHI-3B D$^-$ subline were unresponsive to sera from PBS or MVE-2–treated mice with respect to terminal differentiation (Table 2) or growth inhibition (Fig 2). Conditioned media from BMC or Mφ of MVE-2–treated mice also failed to induce either differentiation (Table 3) or growth inhibition of WEHI-3B D$^-$ cells (data not shown). Likewise, WEHI-3B D$^-$ cells failed to respond to postendotoxin serum (data not shown). Heating of the sera and CM of Mφ and BMC from MVE-2–treated mice (56 °C and 30 min) did not change their stimulatory effect on normal myelopoiesis[2] or their differentiation-inducing activity on WEHI-3B D$^+$ cells (data not shown), suggesting that CSF inhibitors were not present in these sera or CMs. We also incubated WEHI-3B D$^+$ and D$^-$ cells in agar and in liquid suspension cultures directly with MVE-2, at concentrations ranging from 1 to 100 μg/mL to rule out any direct cytotoxic effects of the agent. MVE-2 at these concentrations had no significant growth inhibitory effect and did not induce differentiation of the leukemic cells (data not shown).

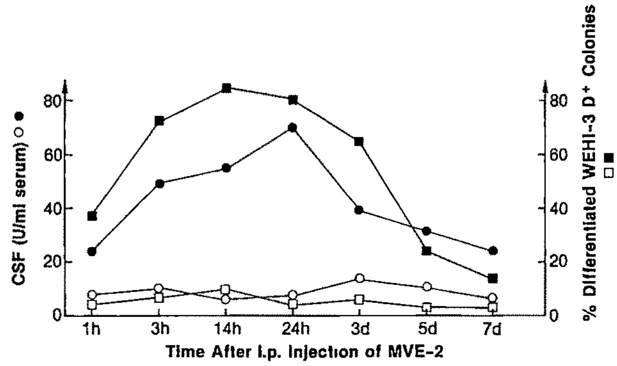

Fig 1. BALB/c mice received a single IP injection of either PBS (open symbols) or 25 mg/kg of MVE-2 (closed symbols). The mice were killed at the times indicated and their sera were harvested for determination of CSF concentrations and for induction of differentiation of WEHI-3B D$^+$ cells. The results are expressed as units of CSF/mL serum or % differentiated WEHI-3B D$^+$ colonies (at a serum dilution of 1/8) and are the mean values of a typical experiment (SD was less than 20%). The experiment was repeated two times and yielded similar results. Incubation of WEHI-3B D$^-$ cells with sera from PBS- or MVE-2–treated mice resulted in up to 5% differentiated WEHI-3B D$^-$ colonies (data not shown).

Table 2. Differentiation-Inducing Activity of Sera From MVE-2–Pretreated Mice on WEHI-3B Leukemia Cells

Group*	Serum Dilution	Percent Differentiated Colonies of WEHI-3B D$^-$ Tumor Cells†	Percent Differentiated Colonies of WEHI-3B D$^+$ Tumor Cells†
Control	1/4	2	11
	1/8	3	9
	1/16	0	4
	1/32	2	3
	1/64	1	3
MVE-2	1/4	8	91
	1/8	5	85
	1/16	2	63
	1/32	0	26
	1/64	3	14
Medium control‡	—	1	2

*Groups of 20 mice were injected IP with either PBS or 25 mg/kg MVE-2. Serum was collected and pooled 14 hours after injection.

†300 WEHI-3B leukemia cells were plated in 1 mL 0.3% agar containing 0.1 mL serum at different dilutions. Each value represents the mean percentage of differentiated colonies of duplicate cultures; SD was less than 20% of the mean. The experiment was repeated 2 times with similar results.

‡300 WEHI-3B leukemia cells were plated in 1 mL of 0.3% agar containing 0.1 medium

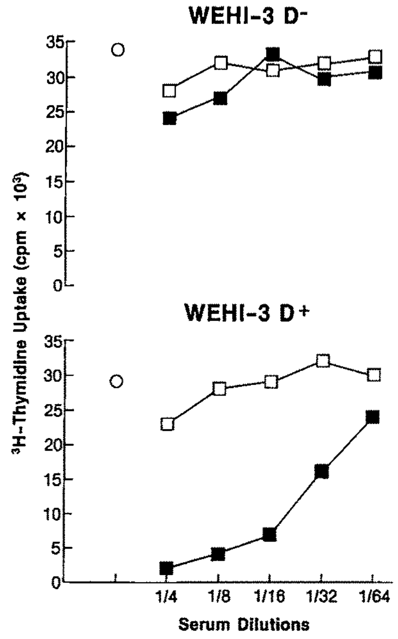

Fig 2. Groups of 20 mice were injected IP with either PBS (□) or 25 mg/kg of MVE-2 (■). Serum was collected and pooled 14 hours after injection. 10^3 WEHI-3B leukemia cells (D^+ or D^-) were then plated in 100 μL of complete medium in 96-well plates. 100 μL of complete medium (○), control serum (□), or serum from MVE-2-treated mice (■) at different dilutions were also added. The cultures were then incubated for 72 hours at 37 °C and 5% CO_2. During the last 6 hours of incubation, they were pulsed with 1 μCi ^{3}H-thymidine/well. The results are the mean of a typical experiment (6 replicates, SD less than 15%) and were repeated twice with similar results.

Table 3. Differentiation-Inducing Activity of Mϕ-CM and BMC-CM From MVE-2-Pretreated Mice on WEHI-3B Leukemia Cells

		Percent Differentiated Colonies of			
		WEHI-3B D⁻ Tumor Cells†		WEHI-3B D⁺ Tumor Cells†	
Group*	CM Dilution	Mϕ-CM	BMC-CM	Mϕ-CM	BMC-CM
Control	1/4	5	2	8	5
	1/8	3	1	6	6
	1/16	4	3	2	3
	1/32	2	2	3	2
	1/64	3	1	3	2
MVE-2	1/4	6	5	94	85
	1/8	2	5	82	89
	1/16	3	1	71	57
	1/32	3	2	34	27
	1/64	2	2	19	12
Medium control‡		3	2	2	4

*Groups of 20 mice were injected IP with either PBS or 25 mg/kg MVE-2. Peritoneal Mϕ and nucleated BMC were collected 14 hours after injection and conditioned media (CM) were prepared by incubating 10^6 Mϕ/mL or 10^7 BMC/mL for 2 days in complete medium without any stimulus.

†300 WEHI-3B leukemia cells were plated in 1 mL 0.3% agar containing Mϕ-CM or BMC-CM at different dilutions. Each value represents the mean percentage of differentiated colonies of duplicate cultures; SD was less than 20% of the mean. The experiment was repeated 2 times with similar results.

‡300 WEHI-3B leukemia cells were plated in 1 mL of 0.3% agar containing 0.1 mL medium.

Combined treatment of WEHI-3B D^+ or D^- tumor-bearing mice with CY and MVE-2. We then wanted to determine whether MVE-2 could be used in vivo, either alone or in combination with CY, for specific treatment of mice bearing WEHI-3B D^+ cells.

Inoculation of BALB/c mice IP with 10^5 WEHI-3B (D^+ or D^-) leukemic cells led to the development of ascitic tumors. The medium survival time (MST) of the tumor controls was about 19 to 20 days with no surviving animals, regardless of the subline inoculated (Figs 3 and 4). Subsequent incubation of WEHI-3B (D^+ + D^-) cells, harvested by peritoneal lavage from the tumor controls 8 days after tumor inoculation in agar cultures, resulted in a 3% to 5% spontaneous differentiation (Table 4).

Treatment of animals bearing the WEHI-3B D^+ subline with MVE-2 (25 mg/kg), 3 and 6 days after tumor inoculation, increased the MST to day 27 (+35% above controls; Fig 3). MVE-2 also induced differentiation in 26% of the remaining peritoneal tumor cells (Table 4). A single IP injection of CY (150 mg/kg) 3 days after tumor inoculation, prolonged the MST to day 28.5 (+ 42% above controls; Fig 3) and induced differentiation in 14% of the remaining peritoneal tumor cells (Table 4). No surviving animals are found after treatment with either MVE-2 or CY alone. Combined treatment with CY (at day +3) and MVE-2 (at day +6), however, delayed MST to day 47.5 (+ 137% above controls), with 30% of the animals still disease free, when the experiment was terminated 90 days after tumor inoculation (Fig 3). The percent survivors after such combined CY/MVE-2 treatment varied in the different experiments between 20% and 50%. The combined CY/MVE-2 treatment also induced differentiation in 61% of the peritoneal tumor cells remaining at day 8 (Table 4).

MVE-2 injections (25 mg/kg) of animals bearing the WEHI-3B D^- subline on days 3 and 6 after tumor inoculation increased the MST of these animals to day 24 (+ 26% above controls), while a single IP injection of CY (150 mg/kg) on day 3 delayed their MST to day 28 (+ 47% above controls; Fig 4). Combined treatment of these mice with CY (at day + 3) and MVE-2 (at day + 6) prolonged MST to day 35, which was an increase of 84% above the MST of the controls (Fig 4). However, neither treatment with MVE-2 or CY alone nor combined treatment with CY/MVE-2 given at a 3-day interval resulted in any surviving animals (Fig 4). Furthermore, none of the treatment regimens used induced differentiation of the peritoneal WEHI-3B D^- tumor cells remaining at day 8 (Table 4).

We then decided to treat tumor-bearing animals with CY/MVE-2 at a one-day time interval (CY at day 3 and MVE-2 at day 4), which does not result in increased CSF levels in vivo (Table 1).

The results in Table 5 show that such a treatment delayed the MST of animals bearing the WEHI-3B D^- or D^+ subline to 32 or 33.5 days, respectively, and no survivors were found. Furthermore, combined treatment with CY on day 3 and

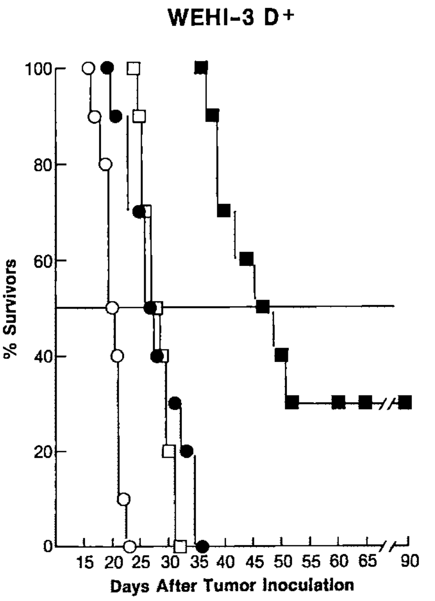

Fig 3. BALB/c mice were inoculated IP with 10⁵ WEHI-3B D⁺ leukemic cells on day 0. Controls (○) were injected IP on day +3 and day +6 with PBS. Some of the mice received eitehr 25 mg/kg of MVE-2 IP on day +3 and day +6 (●) or 150 mg/kg of CY on day +3 (□). Combined treatment with CY (150 mg/kg IP) and MVE-2 (25 mg/kg IP) was administered on day +3 (CY) and day +6 (MVE-2) following tumor inoculaton (■). The mice were monitored for tumor growth, terminal differentiation of tumor cells, and 10 mice/group were kept for median survival time. The results are the mean of a typical experiment, which was repeated 3 times with similar results. The percent of survivors after combined CY/MVE-2 treatment varied in the different experiments between 20% and 50%.

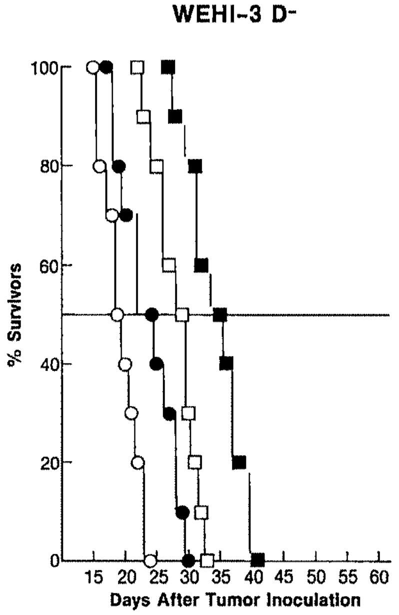

Fig 4. BALB/c mice were inoculated IP with 10⁵ WEHI-3B D⁻ leukemic cells on day 0. Controls (○) were injected IP on day +3 and day +6 with PBS. Some of the mice received either 25 mg/kg of MVE-2 IP on day +3 and +6 (●) or 150 mg/kg of CY on day +3 (□). Combined treatment with CY (150 mg/kg IP) and MVE-2 (25 mg/kg IP) was administered on day +3 (CY) and day +6 (MVE-2) following tumor inoculation (■). The mice were monitored for tumor growth, terminal differentiation of tumor cells, and 10 mice/group were kept for median survival time. The results are the mean of a typical experiment, which was repeated 3 times with similar results.

MVE-2 on day 4 induced a differentiation only in 17% of the peritoneal WEHI-3B D$^+$ cells remaining at day 8, which was not different from the CY control (14%; data not shown). No differentiation was found in the remaining WEHI-3B D$^-$ tumor cells (data not shown).

The residual undifferentiated peritoneal WEHI-3B D$^+$ tumor cells, after treatment with either MVE-2, CY, or CY combined with MVE-2, retained their sensitivity to subsequent in vitro induction of differentiation by postendotoxin serum when tested at day 8, whereas the residual peritoneal WEHI-3B D$^-$ tumor cells remained insensitive to postendotoxin serum with regard to in vitro differentiation or growth inhibition, suggesting these subline are stable (data not shown).

Susceptibility of WEHI-3B D$^+$/D$^-$ leukemic cells to cytotoxicity by Mϕ or NK cells. Since MVE-2 is reported to increase cytotoxicity by Mϕ and NK-cells against various tumor targets[3,4] and treatment with MVE-2 alone or CY combined with MVE-2 was still effective in treating mice bearing the (CSF-insensitive) WEHI-3B D$^-$ subline, we wanted to test whether both tumor sublines were susceptible to cytotoxic Mϕ and/or NK cells.

Both sublines of WEHI-3B (D$^+$/D$^-$) were highly sensitive to Mϕ cytotoxicity at E:T ratios of ≥5:1, whereas they were insensitive to NK-cell cytotoxicity at E:T ratios of ≤200:1 (Table 6). MBL-2 tumor cells were internal positive controls

Table 4. In Vivo Induction of Differentiation of WEHI-3B Leukemia Cells by Combined Treatment with Cyclophosphamide and MVE-2

		Percent Differentiated Colonies of	
Group	Treatment*	WEHI-3B D⁻ Tumor Cells†	WEHI-3B D⁺ Tumor Cells†
1	Control	3	5
2	MVE-2 (25 mg/kg), days 3 and 6	2	26
3	CY (150 mg/kg); day 3	4	14
4	CY (150 mg/kg) on day 3 and MVE-2 (25 mg/kg) on day 6	5	61

*BALB/c mice received on day 0 10⁵ WEHI-3B D⁻ or WEHI-3B D⁺ leukemia cells IP Controls were injected IP on day +3 and day +6 with PBS. Mice from group 2 received 25 mg/kg of MVE-2 IP on day +3 and day +6. Mice from group 3 and 4 were injected on day +3 with 150 mg/kg cyclophosphamide and on day +6 with either PBS or 25 mg/kg MVE-2. All mice were killed on day +8. Their peritoneal cavity was lavaged with 10 mL complete medium and the peritoneal fluid, containing peritoneal exudate cells and WEHI-3B leukemia cells, was harvested. The Mϕ were separated by plastic adherence and the nonadherent WEHI-3B cells were used for determination of their differentiation status.

†300 leukemia cells were plated in 1 mL of 0.3% agar without addition of any sera. Each value represents the mean percentage of differentiated colonies of duplicate cultures, initiated from 5 mice per group; SD was less than 25% of the mean. The experiment was repeated two times with comparable results.

Table 5. MST of WEHI-3B Tumor-Bearing Mice After Combined CY/MVE-2–Treatment Given at a One-Day Time Interval

		MST (days)	
Group	Treatment*	WEHI-3B D^- Tumor Cells	WEHI-3B D^+ Tumor Cells
1	Control	20	19
2	CY (150 mg/kg); day 3	28	29
3	CY (150 mg/kg) on day 3 and MVE-2 (25 mg/kg) on day 4	32	33.5

*BALB/c mice received on day 0 10^5 WEHI-3B D^+ or D^- leukemic cells. Controls were injected IP on day +3 and day +4 with PBS. Mice from groups 2 and 3 received 150 mg/kg of CY on day +3 and PBS (group 2) or 25 mg/kg of MVE-2 (group 3) on day +4. The mice were monitored for tumor growth, terminal differentiation of tumor cells, and 10 mice/group were kept for median survival time (MST). The results are the mean of a typical experiment, which was repeated 3 times with similar results

for Mϕ cytotoxicity[20] and YAC-1 tumor cells[25] for NK-cell cytotoxicity (Table 6).

DISCUSSION

The data presented here show that a chemically defined BRM, MVE-2, may induce in vivo terminal differentiation of malignant myelopoietic progenitor cells, in addition to stimulating growth and differentiation of normal myelopoietic progenitors. These effects on normal or malignant myelopoietic cells, however, appear not to be caused directly by MVE-2, but rather to be mediated through CSFs. This is supported by the observation that MVE-2 has no direct stimulatory or inhibitory effects on normal bone marrow cells grown in soft agar with or without addition of CSF.[2,23] Furthermore, MVE-2 failed to directly induce in vitro differentiation and/or growth inhibition of WEHI-3B D^+ cells. On the other hand, sera or supernatants from BMC and Mϕ of mice pretreated in vivo with MVE-2, which contained different CSFs (these data and reference 2), caused differentiation of malignant myelopoietic progenitor cells. A carry over of MVE-2 with serum to our in vitro cultures can be excluded, since pharmacokinetic studies on the distribution of MVE-2 after in vivo treatment showed that MVE-2 has a short serum half-life.[26]

Although the CSF molecules in serum and supernatants of BMC and Mϕ were not purified, the morphology of the bone marrow colonies induced by these fluids seem to point out[27–29] that sera- and BMC-derived supernatants contained three types of CSF, namely M-CSF, GM-CSF, and G-CSF, as evidenced by the findings of pure Mϕ colonies, mixed granulocyte-Mϕ colonies, and pure granulocyte colonies.[2] Mϕ-derived supernatants contained only M-CSF and G-CSF, as evidenced by the outgrowth of pure Mϕ and pure granulocyte colonies.[2]

While it seems to be clear that CSFs are the major regulator of normal myelopoiesis,[30,31] the problem of whether CSFs are also the major physiologic inducer of differentiation in myelomonocytic leukemias, such as WEHI-3B D^+ cells, is not yet solved. There is experimental evidence that, besides CSFs, there exist factor(s), such as MGI-2[32,33] (also referred to as MGI,[34] D. factor,[35] and GM-DF[11]), which seem to induce differentiation of myelomonocytic cells, while supposedly lacking growth regulatory effects on normal myelopoiesis. However, in conflict with this, others have suggested that GM-DF for myeloid leukemic cells might be identical with G-CSF, inducing growth and differentiation of normal and terminal differentiation of malignant myeloid cells [11,13,16] In our experiments, sera, Mϕ, and BMC supernatants of MVE-2–pretreated mice contained the relevant subclasses of CSF (GM-CSF and G-CSF), which have been shown to induce in vitro differentiation of WEHI-3B D^+ cells,[7,13,14] while lacking such effects on WEHI-3B D^- [these data and reference 17]. The fact that these activities on normal and malignant myeloid progenitors of sera and Mϕ/BMC supernatants from MVE-2–pretreated mice, which were free of CSF inhibitors, showed a parallel time course would support the notion that relevant CSFs present in these fluids (GM-CSF and/or G-CSF) caused the terminal differentiation of the WEHI-3B D^+ cells. This does not, however, exclude the possibility of the concomitant presence of a separate differentiating factor.

The observed therapeutic effects after treatment of mice bearing the WEHI-3B D^+ or WEHI-3B D^- subline with CY

Table 6. Susceptibility of WEHI-3B D^+/D^- Leukemic Cells to Macrophage and NK-Cell Cytotoxicity

MVE-2* (25 mg/kg IP)	Tumor Targets	% Mϕ Cytotoxicity†			% NK Cell Cytotoxicity‡		
		10·1	5.1	1:1	200 1	100 1	50 1
–	WEHI-3B D^-	0	0	0	1.2	−0 2	0 8
+		87§	36§	3	6.1	5.1	3.1
–	WEHI-3B D^+	0	0	0	0.4	0.5	0.4
+		82§	33§	5	3.2	2 6	2.7
–	MBL-2	0	0	0	2.4	1.6	1.0
+		86§	39§	5	4 6	3.3	2 9
–	YAC-1	0	0	0	7.3	5.1	3 3
+		79§	26§	2	46.5§	35.0§	20.6§

*BALB/c mice received on day 0 a single IP injection of either PBS or 25 mg/kg MVE-2. All mice were killed on day +3 for determination of peritoneal Mϕ and spleen NK-cell cytotoxicities against different tumor targets. Each value represents the mean of two separate experiments; SD was less than 20% of the mean.

†Mϕ cytotoxicity of peritoneal Mϕ was determined at different E:T ratios.

‡NK cell cytotoxicity of splenic lymphocytes was determined at different E:T ratios in a 4 hour ^{51}Cr-release assay.[26]

§$P < 0\ 001$ relative to controls

and/or MVE-2 could be due to either one or a combination of the following mechanisms. (1) direct cytotoxic effects of CY on either subline; (2) Mϕ-mediated cytotoxicity on cells of either subline, and (3) CSF-mediated differentiation of the WEHI-3B D$^+$, but not the WEHI-3B D$^-$ subline. Since both sublines have the same origin,[17] are comparable in their biologic behavior (except the induction of differentiation in WEHI-3B D$^+$ cells),[17] and were equally sensitive to Mϕ-mediated cytotoxicity and CY toxicity, the differences in MST between both sublines, treated with MVE-2 alone or a combination of CY and MVE-2, are therefore most likely attributable to CSF-induced differentiation of WEHI-3B D$^+$ cells. This hypothesis is supported by the finding of an increased percentage of differentiated WEHI-3B D$^+$ cells after preceding in vivo therapy with MVE-2 alone or a combination of CY and MVE-2, given at an interval, which allows in vivo induction of CSF.

Treatment of either subline with MVE-2 alone was marginally effective, probably due to the fact that treatment commenced only on day 3, when the IP tumor burden already exceeded 6×10^5 tumor cells (data not shown). We therefore decided to dramatically reduce the tumor burden by CY to a stage of minimal disease before using MVE-2 as Mϕ activator and differentiation-inducing agent. Since our studies have shown that an interval of about 3 days between CY and MVE-2 was optimal for CSF induction and reconstitution of CY-depressed myelopoiesis (these data and reference 36), and regrowth of CY-treated WEHI-3B D$^+$ or D$^-$ cells did not occur until about 4 days after CY (data not shown), we elected a 3-day interval for MVE-2 treatment after preceding high-dose CY. The combined CY/MVE-2 treatment given at a 3-day interval of WEHI-3B D$^-$ tumor-bearing mice significantly increased their MST by about 84% above controls, most likely due to cytoreduction by CY and also more effective Mϕ-mediated cytotoxicity at the minimal residual disease stage (ie, higher Mϕ effector to target cell ratio). The successful treatment, however, of mice bearing the WEHI-3B D$^+$ subline with CY/MVE-2, when given at a 3-day interval (137% increase in MST and 20% to 50% cured mice), seems to be correlated not only with the reduced tumor burden (CY and Mϕ-mediated), but also with the induction of differentiation in remaining tumor cells, which was in a high percentage of the cases terminal, thus preventing further leukemogenesis.[33,37] This is supported by the findings that treatment of mice bearing WEHI-3B tumor cells with a combination of CY and MVE-2, given at a one-day interval, which does not allow the in vivo induction of CSF, resulted only in a rather small increase in MST with no surviving animals; also no differences were found between the WEHI-3B D$^+$ and D$^-$ sublines. Furthermore, MVE-2 given one day after CY failed to induce differentiation in the remaining WEHI-3B D$^+$ cells. The finding that the residual undifferentiated WEHI-3B D$^+$ tumor cells after treatment with CY/MVE-2 remain sensitive to differentiation with CSF seems to rule out the possibility of an immediate in vivo selection process for WEHI-3B D$^-$ variants, which would preclude further cyclic treatment with CY/MVE-2.

Our studies thus show that combined treatment of WEHI-3B D$^+$ tumor-bearing mice with CY and the BRM, MVE-2 depend on an optimal treatment schedule, which takes into account the time course of the cytoreductive effects of CY on the tumor cells, as well as the modification of the host's immune system. Furthermore, MVE-2 offers the distinct advantage of being a potent CSF inducer, which in turn has profound effects on CY-depressed myelopoiesis and terminal differentiation of certain myelomonocytic tumor cells. Even though it is unlikely that the results obtained with the differentiation-inducible murine WEHI-3B D$^+$ leukemia can be extrapolated to the majority of primary murine or human myelomonocytic leukemias, our results and those of others[33,38] clearly indicate that biologic response modification may occur in vivo leading to phenotypic changes of tumor cells, thus possibly eliminating the growth advantage of leukemic tumor cells relative to the normal myelomonocytic stem cell population[39] and/or preventing further leukemogenesis.[33,37]

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Mechanisms of Adenosine 5'-Monophosphate Catabolism in Human Erythrocytes

By Donald E. Paglia, William N. Valentine, Misae Nakatani, and Richard A. Brockway

Uncertainties regarding the role of pyrimidine nucleotidase (PyrNase) in AMP catabolism were resolved by studies of erythrocytes from normal controls, controls with young mean cell ages, and patients with hereditary hemolytic anemia due to severe deficiency of PyrNase. Hemolysates from the latter exhibited undiminished capacity to dephosphorylate AMP over a broad range of pH, indicating that PyrNase was not directly involved. In each subject group, the rates of AMP dephosphorylation between pH 5.1 and 8.3 were indistinguishable from those of IMP, suggesting a potential role for AMP-deaminase, an erythrocyte enzyme that was stimulated by coformycin at pH 7.2. Quantitative analysis of catabolites in incubated hemolysates confirmed that AMP degradation preferentially occurred via deamination to IMP with subsequent dephosphorylation by another erythrocyte nucleotidase isozyme, deoxyribonucleotidase. Both AMP-deaminase and deoxyribonucleotidase have acidic pH optima with minimal activities at physiologic pH, suggesting that this pathway of AMP catabolism could accelerate depletion of the adenine nucleotide pool and thereby mediate the demise of senescent erythrocytes sequestered in the spleen.

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NUCLEOTIDASES (5'-ribonucleotide phosphohydrolase, E.C.3.1.3.5) comprise an enzyme class that is common to microorganisms and to most plant and animal tissues. Individual isozymes dephosphorylate both purine and pyrimidine 5'-monophosphates with variable effectiveness, but human erythrocytes contain an isozyme (pyrimidine nucleotidase, PyrNase) uniquely restricted to pyrimidine substrates.[1,2] Such substrate specificity presumably preserves adenosine 5'-monophosphate (AMP), which is in equilibrium with the di- and triphosphates (ADP, ATP) essential to normal cellular function and longevity.

Under some in vitro circumstances, human hemolysates and partially purified enzyme preparations appear to degrade AMP actively. This has been attributed to PyrNase activity, but may actually represent alternative catabolic pathways. One such possibility, conversion of AMP to inosine 5'-monophosphate (IMP) by AMP-deaminase with subsequent dephosphorylation by another nucleotidase isozyme, deoxyribonucleotidase (dNase), is supported by data in the present study.

MATERIALS AND METHODS

The ability of hemolysates to liberate inorganic phosphate (P_i) from AMP and other purine and pyrimidine 5'-monophosphate substrates was assessed by modification[3] of a technique described previously.[1] Nucleotidase activities as a function of pH were determined on 14 blood samples from 13 normal controls, on samples from nine subjects with reticulocytosis (4% to 23%, mean = 8.5%) but with no identifiable enzyme defect, and on six samples from three subjects with hemolytic anemia secondary to severe (<5%) hereditary deficiency of PyrNase (7% to 12% reticulocytes, mean = 10.5%).

Saline suspensions of washed erythrocytes prepared by internationally standardized techniques[4] were lysed by sonication and dialyzed free of endogenous phosphates against 200 volumes of isotonic saline buffered to pH 8.0 with 10 mmol/L Tris-HCl containing 10 mmol/L $MgCl_2$, 0.02 mmol/L EDTA, and 1 mmol/L mercaptoethanol at 4 °C. Hemolysates were redialyzed against fresh solutions for 2 hours prior to assay. Aliquots of dialyzed hemolysate were incubated at 37 °C in 0.15 mmol/L Tris-maleate buffer with 8.5 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol, and 2.3 mmol/L AMP or other substrates. Final pH of each incubation mixture was varied at eight points between pH 5.1 and 8.3 and was measured both before and after the 2-hour incubation period with a Beckman Model pHI 41 digital pH meter calibrated against Beckman standard phosphate buffers. Reactions were terminated by addition of equal volumes of 1.2 N perchloric acid. Inorganic phosphate was measured by the Fiske and SubbaRow technique.[5] Reagent blanks were incubated with hemolysate alone, the appropriate substrate being added after acid deproteinization

To measure AMP or dAMP degradation products directly, dialyzed hemolysate was buffered with either 25 mmol/L Tris-HCl, pH 7.4, or 0.25 mol/L potassium succinate, pH 6.2, containing 8 mmol/L $MgCl_2$, 0.6 mmol/L dithiothreitol, 2.2 mmol/L AMP or dAMP, with or without 0.04 mmol/L coformycin Final pH was 7.2 (Tris-HCl) or 6.2 (succinate).

Reactions were terminated by heating to 100 °C for 10 minutes. Supernates, filtered free of coagulum, were assayed for residual substrate and degradation products by enzymatic techniques.[6,7] Aliquots of deproteinized supernate were buffered with 0.1 mol/L Tris-HCl, pH 8.0, containing 0.5 mmol/L EDTA, 2 mmol/L $MgCl_2$, and 0.05 mmol/L sodium phosphate. Addition of 0.005 mL commercial preparations of xanthine oxidase, nucleoside phosphorylase, 5'-nucleotidase, alkaline phosphatase, and adenosine deaminase in various sequences and combinations allowed measurement of hypoxanthine, inosine/deoxyinosine, IMP/dIMP, and adenosine/deoxyadenosine by monitoring absorbance at 293 nm.[6,7]

For ease of comparison, nucleotidase activities and generation of specific reaction products are expressed in common units (U), micromoles produced per hour per gram of hemoglobin (Hgb). Although these are expressed as reaction rates, they may more closely approximate equilibrium concentrations of metabolites in some prolonged incubations that have high initial reaction rates and consequent substrate depletion.

RESULTS

Regardless of whether subjects were normal controls, controls with reticulocytosis, or patients with severe PyrNase deficiency, their incubated hemolysates generated P_i from AMP and IMP at virtually identical rates. As shown in Fig 1, this occurred throughout the entire pH range studied (pH

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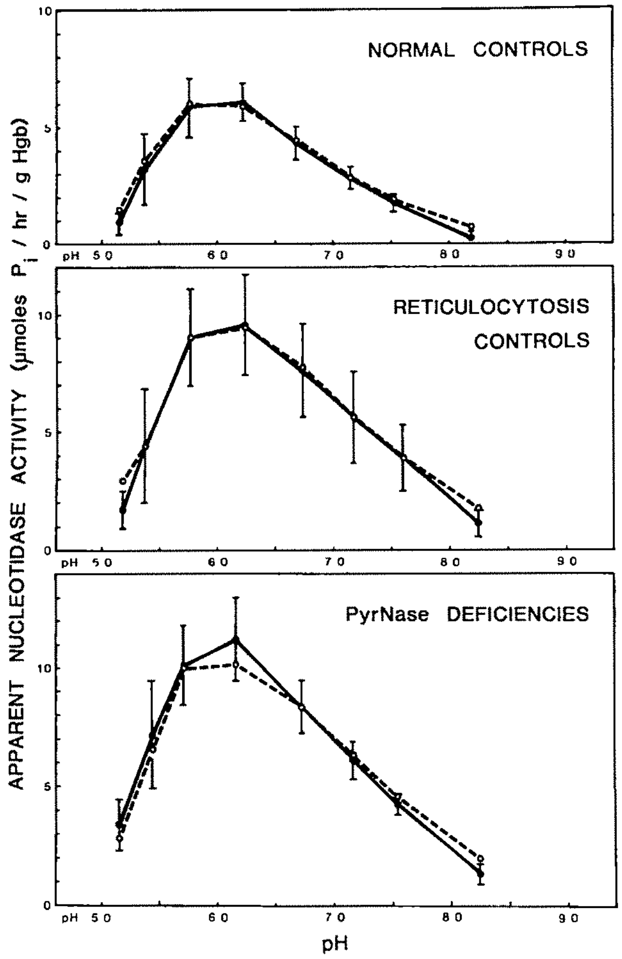

Fig 1. Inorganic phosphorous generation from AMP (solid curves) and IMP (broken curves) as a function of pH. Each point is the mean of multiple assays on 6 to 14 specimens from 3 to 13 subjects as described under Methods. Brackets indicate ±1 SD around mean values for AMP.

5.15–8.25). Subjects with reticulocytosis exhibited predictably greater activities than normal controls, since most enzymes, including PyrNase,[1,2] are more active in young erythrocytes than in older cohorts. There was no apparent difference in P_i production from AMP between subjects with PyrNase deficiency and controls with reticulocytosis, indicating that erythrocyte PyrNase was not the source of AMP dephosphorylation under these conditions.

Similar pH-optima curves for IMP and dIMP, shown in Fig 2, indicate that these nucleotides are also ineffective substrates for PyrNase. Erythrocytes from a patient with severe PyrNase deficiency showed greater activities with IMP and dIMP than did 10 normal controls or 5 to 11 subjects with reticulocytosis (latter values not shown in Fig 2). Similarities in pH optima for IMP and dIMP, coupled with major differences in their relative activities, may be an expression of variable substrate preference (cross-reactivity) of erythrocyte dNase, which is optimally active at pH 6.2.[3]

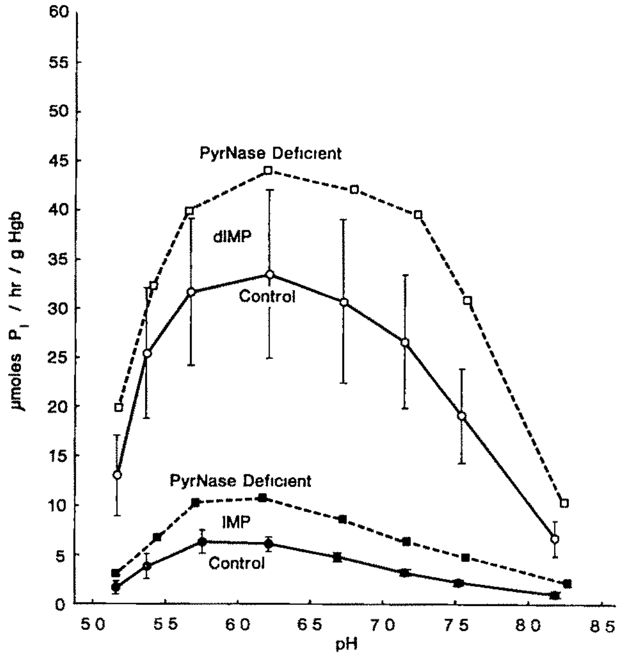

Fig 2. Inorganic phosphate generation from IMP (solid symbols) and dIMP (open symbols) as a function of pH. Each point is the mean of 10 determinations with normal control hemolysates (solid curves) or two assays of duplicate specimens from a single subject with severe hereditary PyrNase deficiency (broken curves). Brackets indicate ± 1 SD around control curves.

Identical rates of P_i production from AMP and IMP over a broad range of pH strongly suggested that erythrocyte AMP-deaminase might convert AMP to IMP prior to dephosphorylation. This hypothesis was supported by in vitro incubation experiments with normal control erythrocytes, which are summarized in Table 1. When hemolysates were incubated at pH 7.2 under conditions commonly used for assay of PyrNase activity, AMP was preferentially converted to IMP (5.8 U) rather than into adenosine (undetectable) and P_i (0.8 U). (The sensitivity of the assay system is such that values in the region of 0.5 U or below are negligible and

Table 1. Degradation Products of AMP and dAMP Incubated with Normal Control Hemolysates

		AMP Degradation Products (µmol/hr/g Hgb)						dAMP Degradation Products (µmol/hr/g Hgb)					
pH	Coformycin	IMP	Adenosine	Inosine	Hypoxanthine	P_i	Total Purines	dIMP	d-Adenosine	d-Inosine	Hypoxanthine	P_i	Total Purines
7.2	0	5 8	ND*	0.8	0.6	0.8	7.2	ND	0.6	0.8	ND	0.5	1.4
7.2	+	26 6	ND	1.0	0.9	0.9	28.5	ND	1.2	ND	0.6	1.3	1.8
6.2	0	20.4	ND	6.6	1.3	7.1	28.3	ND	1 4	7.3	1.6	8.4	10.3
6 2	+	21.4	2 5	5 5	1.2	7.1	30.6	ND	7.6	1.6	0.7	8.8	9.9

Values are means derived from six (dAMP) or seven (AMP) separate experiments.

*ND = not detectable.

not reliably reproducible). Traces of inosine (0.8 U) and hypoxanthine (0.6 U), if real, may have derived from subsequent degradation of either adenosine (via adenosine deaminase) or IMP (via dNase) (Fig 3). The latter would seem more likely, since inhibition of adenosine deaminase with coformycin did not result in adenosine accumulation (Table 1).

In six paired experiments at pH 7.2, dIMP was not produced in detectable quantities from dAMP, whereas IMP production from AMP was abundant (5.8 to 26.6 U). Other potential dAMP degradation products (deoxyadenosine, deoxyinosine, hypoxanthine, and P_i) were also sparse. This is consistent with the observation that erythrocyte AMP-deaminase cannot utilize dAMP as substrate.[6] At pH 6.2, however, significant quantities of purine degradation products and P_i were generated from dAMP in almost equivalent amounts, consistent with erythrocyte dNase activity, which is optimal at pH 6.2.[3]

As an incidental observation, the presence of coformycin at pH 7.2 increased the yield of IMP from AMP fourfold (Table 1). This was an invariable effect in each of the seven paired hemolysates incubated with AMP. At pH 6.2, similarly high yield of IMP occurred regardless of the presence or absence of coformycin. No comparable differential in dIMP production from dAMP was induced by coformycin at either pH 7.2 or 6.2.

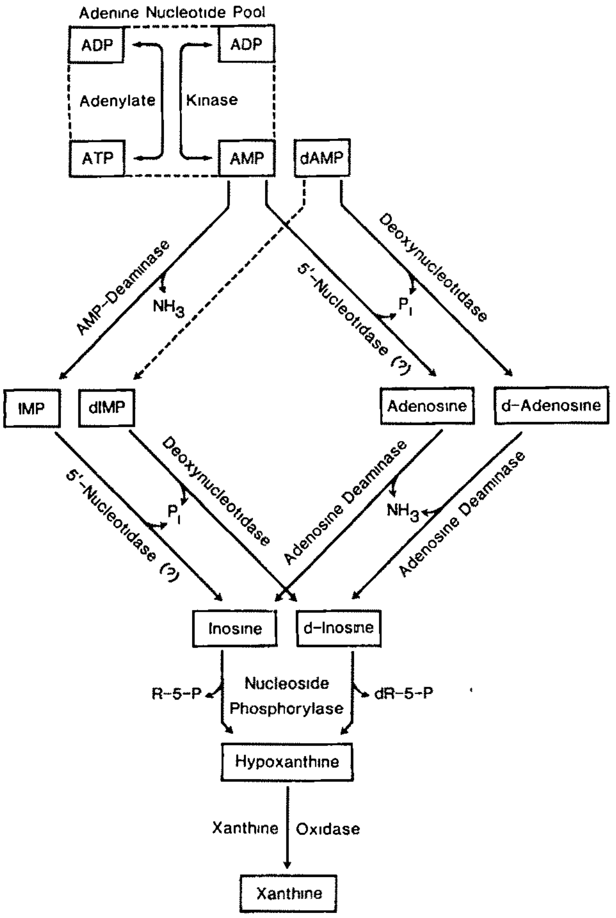

Fig 3. Potential pathways of AMP and dAMP catabolism in human erythrocytes. Erythrocyte AMP-deaminase is inactive with dAMP. Pathways designated "5'-Nucleotidase (?)" are more likely mediated by deoxyribonucleotidase cross-reacting with nondeoxy substrates. Coformycin blocks the adenosine deaminase reaction.

DISCUSSION

Erythrocytes depend on energy stored in terminal phosphate bonds of ATP to fuel a number of metabolic reactions essential to normal function and longevity. Although AMP constitutes only 1% to 3% of the total adenine nucleotide pool, it occupies a pivotal position in the metabolic economy of the cell.

First, it is in rapid equilibrium with the other nucleotide components, mediated by an active adenylate kinase:

$$\mathrm{AMP} + \mathrm{ATP} \underset{\text{Kinase}}{\overset{\text{Adenylate}}{\longleftrightarrow}} 2\,\mathrm{ADP}$$

Second, formation of AMP from plasma adenosine provides an essential salvage pathway for nucleotide replenishment:[8,9]

$$\text{Adenosine} + \mathrm{ATP} \xrightarrow[\text{Kinase}]{\text{Adenosine}} \mathrm{AMP} + \mathrm{ADP}$$

Third, adenine nucleotides, which cannot be synthesized de novo, may be irretrievably lost by conversion of AMP to nonutilizable metabolites, for example, by deamination:

$$\mathrm{AMP} + \mathrm{H_2O} \xrightarrow{\text{AMP-Deaminase}} \mathrm{IMP} + \mathrm{NH_3};$$

or by degradation to diffusible catabolites, for example, by nucleotidase activity:

$$\mathrm{AMP} + \mathrm{H_2O} \xrightarrow{\text{5'-Nucleotidase}} \text{Adenosine} + \mathrm{P_i}$$

In apparent deference to the latter, many mammalian erythrocytes have evolved nucleotidase isozymes that uniquely exclude AMP from their physiologic substrates.

The existence of a pyrimidine-specific nucleotidase (PyrNase) in human erythrocytes became apparent through investigation of patients with hereditary hemolytic anemia secondary to severe deficiency of that enzyme.[1] Initial studies of PyrNase properties in normal hemolysates indicated that AMP and other purine nucleotides were not effective substrates,[1,2] but that both purine and pyrimidine 2'-deoxyribonucleotides apparently were.[2] Dephosphorylation of some deoxy substrates was also detected in PyrNase-deficient cells[10,11] and subsequently proven to be due to deoxyribonucleotidase (dNase), a normal erythrocyte isozyme (or isozymes) distinct from PyrNase.[3]

In the interim, however, other investigators had observed that AMP could be degraded by hemolysates or partially purified preparations under specific in vitro conditions.[12,13] Resolution of these uncertainties regarding substrate specificity of PyrNase is important to understanding normal erythrocyte metabolism as well as molecular mechanisms of hemolysis in severe PyrNase deficiency states. PyrNase deficiencies are now recognized as among the more common enzymopathies responsible for hereditary hemolytic anemia and also contribute to the acquired anemia induced by lead toxicity.[14] Theoretically, a mutant PyrNase isozyme that also catalyzed dephosphorylation of AMP to diffusible adenosine might predominate over salvage reactions, such as that mediated by adenosine kinase, to cause an inexorable drain on the adenine nucleotide pool. Mechanisms other than mutant PyrNase, however, are required to explain apparent AMP dephosphorylation by normal erythrocytes.[13]

The present study provides some clarification of these conflicting observations. The data clearly establish that AMP is not a substrate for normal erythrocyte PyrNase, since cells from several subjects with severe PyrNase deficiency retained the same capacity to degrade AMP as normal and reticulocyte-rich specimens. This held true for IMP and dIMP, as well as for AMP, over a broad range of pH. These findings have been corroborated by supplementary studies with larger populations, using 12 different purine and pyrimidine ribo- and deoxyribonucleotides, which identified only UMP, CMP, and dCMP as direct substrates for normal erythrocyte PyrNase.[15]

The present study confirmed that AMP degradation may occur in human hemolysates, but that it is mediated by AMP-deaminase and dNase rather than PyrNase. Multiple paired experiments with normal red cells, young cell populations, and PyrNase-deficient cells indicated that rates of P_i evolution from AMP and IMP were virtually identical, regardless of pH (Fig 1). This would be expected if AMP were deaminated to IMP prior to dephosphorylation, an hypothesis that was confirmed by analysis of degradation products in incubated hemolysates (Table 1). Other studies have also shown that AMP-deaminase predominates over nucleotidase activity in certain leukocytes as well as erythrocytes, an imbalance that is pertinent to understanding intracellular accumulations of deoxynucleotides in severe combined immunodeficiency disease.[6]

It is interesting that the presence of coformycin, a potent inhibitor of adenosine deaminase, consistently increased IMP production from AMP more than fourfold at pH 7.2, presumably by stimulation of AMP-deaminase. Comparable increases in AMP-deaminase activity occurred when pH was shifted from 7.2 to 6.2. The mechanisms involved remain unknown but may relate to coformycin- or pH-induced alterations of secondary or tertiary structure of the enzyme protein that serve to increase its substrate avidity and catalytic effectiveness. Additional data, not included under Results, indicated that acidic pH protects both PyrNase and dNase from inactivation by lead, presumably by similar conformational changes that shelter sensitive sulfhydryl groups.

These studies demonstrate that AMP-deaminase and dNase combine to provide an effective pathway for AMP degradation in vitro, but the role of these enzymes in normal cellular metabolism remains unclear. If significant conversion of AMP to IMP were to occur in vivo, it would constitute a hazard equivalent to AMP dephosphorylation, since erythrocytes cannot resynthesize adenine nucleotides from IMP or dIMP[16] nor from the degradation products, inosine and hypoxanthine. A priori, then, this pathway cannot be highly active under normal in vivo circumstances, or the adenine nucleotide pool would be quickly depleted. Additionally, dNase activity is apparently insufficient physiologically to clear deoxyadenine nucleotides from cells of patients with severe combined immunodeficiency disease secondary to adenosine deaminase deficiency. The role of AMP-deaminase in such deoxynucleotide accumulation in the latter condition has been discussed in a separate study.[6]

It is tempting to speculate that the sharply acidic pH optima of AMP-deaminase and dNase suppress this pathway at physiologic pH but allow activation under specific circumstances. In the hypoxic, acidic environment of the spleen, for example, sequestered erythrocytes might progress rapidly from senescence to death via an accelerated drain on the adenine nucleotide pool mediated in part by these two enzymes. We have speculated previously[3,11] that dNase might serve physiologically to clear DNA degradation products from maturing erythroblasts and reticulocytes in a role analogous to that presumed for PyrNase in RNA catabolism.[1,11] If such were the case, it would be ironic that an enzyme essentially functioned only at the two extremes of the cell life span, mediating events both in maturation and demise. Such speculations may palliate the compulsion to seek purpose in Nature's design, but precise physiologic roles will more likely be revealed by future study of patients with specific deficiencies of these enzymes.

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An Explanation for Nonimmunologic Adsorption of Proteins Onto Red Blood Cells: Schiff's Base Reactions

By D. Jamin, J. Demers, I. Shulman, H.T. Lam, and R. Momparler

During a phase I evaluation of diglycoaldehyde (INOX), an intravenous chemotherapeutic agent used to treat children with malignancies, all of eight patients tested developed a positive direct antiglobulin test (DAT) in vivo. The DAT became positive within one to seven days after the first administration of the drug and remained positive for up to 12 days following the last dose. The indirect antiglobulin tests were negative. None of the patients showed clinical or laboratory evidence of hemolysis at the time the DAT became positive or during follow-up. Eluates made from the red cells of two of the eight patients were both negative by indirect antihuman globulin testing. In vitro studies with INOX and glutaraldehyde, both dialdehyde compounds, showed nonimmunologic adsorption of protein onto red cells, probably by the condensation of aldehyde groups of these compounds to form Schiff's bases with amino acids of serum proteins and red cell membrane proteins. This reaction provides an explanation for the globulin detected on the red cells of patients treated with INOX.

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INOX (diglycoaldehyde) is the periodate oxidation product of the purine nucleoside, inosine, in which carbons 2′ and 3′ have been oxidized to formyl groups (Fig 1A).[1,2] In vitro studies have indicated that the formyl groups can bind to proteins by a Schiff's type reaction and that this effect may be partly responsible for the antitumor properties of INOX.[3–5] During a phase I evaluation of INOX, all of eight patients tested developed a positive direct antiglobulin test (DAT). The DAT became positive within one to seven days after the first administration of the drug and remained positive for up to 12 days following the last dose. The indirect antiglobulin tests were negative. The possibility that INOX might have caused a nonimmunologic adsorption of serum proteins onto the red cells of the patients via Schiff's type reactions was investigated in a series of laboratory studies. Glutaraldehyde, a compound that forms Schiff's type reactions with proteins, was included in these studies.

MATERIALS AND METHODS

Patients. Direct antiglobulin tests were performed on the red cells of eight children before, during, and after the intravenous infusion of INOX in doses of up to 1,500 mg/m^2 per day for 4 to 10 days per course. The sex, age, and diagnosis of each patient is recorded in Table 1.

Materials. Routine techniques and commercially available reagents were used.[6] Reagents used included anti-IgG+C3b+C3d+C4 (Immucor, Norcross, Ga), anti-IgG (BCA, Malvern, Pa), anti-C3b+C3d+C4 (Ortho Diagnostics, Raritan, NJ), anti-bovine albumin (Cappel, Cochranville, Pa), anti-human albumin (Cappel), 22% bovine albumin (BCA; 22% bovine albumin was diluted to make 6% bovine albumin), 16 5% human immune serum globulin (Armour Pharmaceutical, Kankakee, Ill), 25% human albumin (Travenol, Glendale, Calif), and glutaraldehyde, 3000 ug/mL (Sigma, St. Louis, Mo). Inosine dialdehyde (INOX, NSC-118994) was supplied as a lyophilized powder by the National Cancer Institute.

Preparation of INOX solution. INOX was reconstituted in phosphate buffered saline (PBS), at either pH 7.0 or 7.4 to a final concentration of 132 mg/dL

Incubation of normal red cells with INOX plus selected protein media. One milliliter of normal cells was incubated at 37 °C for two hours with one mL of INOX, 132 mg/dL, plus one mL of either normal human serum, normal human plasma, 16.5% human immune serum globulin, 25% human albumin, 6% bovine albumin, or 22% bovine albumin. Red blood cells incubated with INOX and normal saline were used as a control. Following the incubation, the red cells were washed three times with normal saline and tested for cell-bound protein by antiglobulin and antialbumin tests using anti-IgG+C3b+C3d+C4, anti-IgG, anti-C3b+C3d+C4, antihuman albumin, and antibovine albumin

Incubation of normal red cells with glutaraldehyde and selected protein media. One milliliter of normal red cells was incubated at 37 °C for one hour with one mL of 300 mg/dL glutaraldehyde (either pH 6 5 or pH 7.4) plus one mL of either normal human serum, normal human plasma, 16.5% human immune serum globulin, 25% human albumin, 6% bovine albumin, or 22% bovine albumin. Red blood cells incubated with glutaraldehyde and normal saline was used as a control. Following the incubation, the cells were washed three times with normal saline and tested for cell-bound protein by antiglobulin and antialbumin tests using anti-IgG+C3b+C3d+C4, anti-IgG, anti-C3b+C3d+C4, antihuman albumin, and antibovine albumin.

Adsorption-elution studies. One milliliter of normal red cells was incubated for two hours at 37 °C with one mL of INOX plus one mL of 25% human albumin. The red cells were then washed six times with PBS (pH 7.4). The washed red cells were added to an equal amount of glycine buffer solution (pH 1.8). An eluate was obtained by spinning down this mixture and recovering the supernatant. Tris(hydroxymethyl)-aminomethane solution was used to neutralize the acidity of the eluate. The eluate pH was adjusted to 7.4 or 6 5 with 0.1N NaOH or 0.1N HCl.

One milliliter of the eluate was incubated with 0.1 mL of normal red cells at 37 °C for two hours. Following the incubation, the cells were washed three times with PBS, pH 7.4, and tested with antihuman albumin. Red cells incubated with human albumin alone and red blood cells incubated with INOX alone were used as controls.

Incorporation of radiolabeled INOX onto red cells. Radiolabeled 14C INOX was supplied by the Stanford Research Institute through Dr R. Engle of the National Cancer Institute. One milliliter each of red blood cells, normal plasma, and INOX-8-14C, at a concentration of 2 mg/mL (4.2 mCi/mmol, 14.2 microCi/mg) were incubated for up to 2 hours at 37 °C. At 5 minutes, 30 minutes, one hour, and two hours, 0.5 mL aliquots of the mixture were diluted with 6 mL of 0.9% NaCl, and centrifuged at 3,000 RPM for 5

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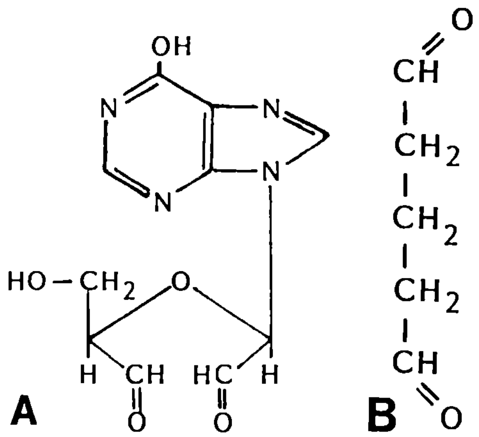

Fig 1. Chemical structures of (A) diglycoaldehyde (INOX) and (B) glutaraldehyde.

Table 2. Antiglobulin and Antialbumin Reactions of Normal Red Cells After Incubation With INOX Plus Selected Protein Media at pH 7.4

	Anti-IgG + C*	Anti-IgG	Anti-C*	Antihuman Albumin	Antibovine Albumin
INOX plus					
0.9% saline	0	0	0	0	0
normal serum	2+	2+	0	1+	—
normal plasma	2+	2+	0	w+	—
16.5% human gamma globulin	16†	16†	0	0	0
25% human albumin	0	0	0	>1024†	—
6% bovine albumin	0	0	0	—	128†
22% bovine albumin	0	0	0	—	128†

*Anti-C3b + C3d + C4

†Greatest dilution of antiserum that gave macroscopic agglutination Normal red cells incubated in 16.5% human gamma globulin, 25% human albumin, or 6% and 22% bovine albumin without the addition of INOX were agglutinated by anti-IgG, antihuman albumin, and antibovine albumin, respectively, at dilutions of 1:2.

minutes. The precipitate was washed with 0.9% NaCl, centrifuged, and the supernatant discarded The washing was repeated two times Five mL of cold 5% trichloracetic acid were then added to the pellet; the mixture was centrifuged, and the supernatant discarded. The precipitate was washed in saline, and then suspended in 1 mL of NCS tissue solubilizer (Amersham) and incubated at 37 °C for 48 hours. A 0.2 mL aliquot of the solubilized material was placed on a GF/C glass fiber filter (2.4 cm in diameter), placed in Omnifluor Scintillation Fluid (New England Corp), and assayed for radioactivity using a Beckman 360 Scintillation counter. Red blood cells incubated with 5% dextrose and INOX were used as a control.

Grading and scoring. All grading of agglutination was in accordance with the technical manual of the American Association of Blood Banks.[6]

RESULTS

All eight patients developed a positive DAT within seven days after the first intravenous dose of INOX (Table 1). Heat prepared eluates were made from the red cells of two of the eight patients. Both of these eluates (pH approximately 7) were negative by indirect antihuman globulin testing. In patient #8 the DAT became positive following the first dose of INOX and in patient #4 remained positive for at least 12 days after the last dose of INOX. None of the patients showed clinical or laboratory evidence of hemolysis at any time; bilirubin concentration, hemoglobin concentration, and reticulocyte counts remained stable.

Table 2 summarizes the results of antiglobulin and antialbumin tests on normal red cells incubated with INOX and either normal human serum, normal human plasma, 16.5% human immune serum globulin, 25% human albumin, 6% bovine albumin, or 22% bovine albumin. The results were positive for all corresponding proteins except complement, and were stronger at pH 7.4 than at pH 7.0.

Table 3 summarizes the antiglobulin and antialbumin test results on normal red cells incubated at pH 7.4 with glutaraldehyde plus either normal human serum, normal human plasma, 16.5% human immune serum globulin, 25% human albumin, 6% bovine albumin, or 22% bovine albumin. The results were positive for all corresponding proteins except

Table 1. Clinical Data

Patient	Sex	Age (yr)	Diagnosis	Dose of INOX ($mg/m^2/d$)	Direct Antiglobulin Test*			
					Before	Positive After Dose		Follow-up
1†	M	2	Neuroblastoma	1000 mg/10 d	Neg	7th	3+‡	+6 days
2†	M	7	Wilms	1000 mg/5 d	Neg	2nd	2+	+2 days
3	M	16	ALL	1500 mg/5 d	Neg	2nd	2+	+8 days
4	F	16	ALL	1500 mg/5 d	Neg	2nd	2+	+12 days
5	M	9	Rhabdomyosarcoma	1500 mg/4 d	Neg	4th	1+	+7 days
6	F	14	Osteogenic sarcoma	1500 mg/5 d	Neg	3rd	1+	+2 days −24 days
7	M	11	Ewing's sarcoma	1500 mg/4 d	Neg	4th	2+	+11 days
8§	M	10	ALL	1500 mg/5 d	Neg	1st	1+	+8 days

*Direct antiglobulin tests were performed on blood samples taken before, during, and after therapy with INOX.

†Negative indirect antiglobulin test with eluates from red cells

‡Maximum strength of DAT.

§Patient was receiving Cephalexin

Table 3. Antiglobulin and Antialbumin Reactions of Normal Red Cells After Incubation With Glutaraldehyde Plus Selected Protein Media at pH 7.4

	Anti-IgG + C*	Anti-IgG	Anti-C*	Antihuman Albumin	Antibovine Albumin
Glutaraldehyde plus					
0.9% saline	0	0	0	0	0
normal serum	2+	2+	0	1+	—
normal plasma	2+	2+	0	w+	—
16.5% human gamma globulin	8†	8†	0	0	0
25% human albumin	0	0	0	>1024†	—
6% bovine albumin	0	0	0	—	>1024†
22% bovine albumin	0	0	0	—	>1024†

*Anti-C3b + C3d + C4.

†Greatest dilution of antiserum that gave macroscopic agglutination Normal red cells incubated in 16.5% human gamma globulin, 25% human albumin, or 6% and 22% bovine albumin without the addition of glutaraldehyde were agglutinated by anti-IgG, antihuman albumin, and antibovine albumin, respectively, at dilutions of 1 2.

complement. When the cells were incubated at pH 6.5, all reactions were negative.

Eluates prepared from red cells that had been incubated with human albumin plus INOX reacted with normal red cells using antihuman albumin when the pH of the eluate was 7.4 but not when the pH was 6.5. Control studies, ie, eluates at pH 7.4 prepared from red cells that had been incubated with human albumin without the addition of INOX were negative as well as acid eluates prepared from red cells that had been incubated with INOX and normal saline.

Fig 2 shows the uptake of radiolabeled INOX onto red blood cells.

DISCUSSION

Many drugs and chemicals are known to induce positive direct antiglobulin tests.[7] Drugs such as penicillin, quinidine, alpha-methyldopa, and cephalosporins have been well documented as causing positive direct antiglobulin tests in patients via a variety of mechanisms. None of these mechanisms is identical to that which is proposed to explain how INOX causes a positive direct antiglobulin test in vivo.

The positive antiglobulin and antialbumin tests induced in these studies demonstrate that INOX and glutaraldehyde can cause the adsorption of proteins onto red cells. The fact that albumin was adsorbed onto red cells from serum, plasma, or human or bovine albumin media in the presence of INOX or glutaraldehyde suggests that the mechanism is nonimmunologic. The proposed mechanism by which INOX and glutaraldehyde cause nonimmunologic adsorption of proteins onto red cells is via the formation of Schiff's bases.

INOX is the periodate oxidation product of the purine nucleoside, inosine, in which carbons 2′ and 3′ have been oxidized to formyl groups (Fig 1A).[1,2] Glutaraldehyde is similar in structure to INOX in that it also contains two free formyl groups (Fig 1B). In vitro studies have demonstrated that formyl groups can bind to proteins by a Schiff's type reaction.[3-5] Glutaraldehyde has previously been described as forming Schiff's type reactions with proteins.[8]

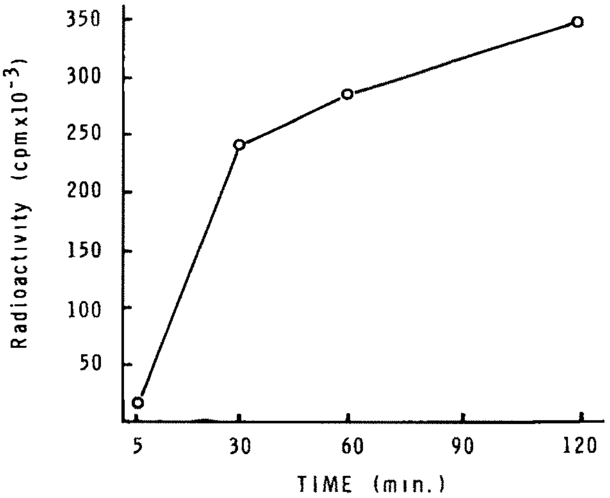

Fig 2. Incorporation of radiolabeled INOX into red blood cells. Radiolabeled INOX (2 mg/mL, 14.2 μCi/mg) was incubated with fresh plasma and red blood cells at 5, 30, 60 and 120 minutes; the radioactivity incorporated into the acid-insoluble fraction of red blood cells was determined.

Our hypothesis is that when red cells are incubated with either INOX or glutaraldehyde in an alkaline medium, one of the aldehyde groups of either INOX or glutaraldehyde forms a Schiff's base with an amino group of the red cell membrane. The other aldehyde group is free to form a Schiff's base with an amino group of a plasma protein. This proposed mechanism can explain the findings of a positive DAT in patients receiving intravenous INOX (pH of blood is 7.4) and the in vitro results described in this report. This hypothesis is supported by the observation that the adsorption of proteins onto red cells was pH dependent. Protein adsorption was detected when glutaraldehyde was used at a pH of 7.4 but not at a pH of 6.5. Less protein adsorption was detected when INOX was used at a pH of 7.0 than of 7.4. INOX and human albumin eluted from red blood cells could be readsorbed onto fresh red cells at a pH of 7 4 but not at a pH of 6.5.

Heat-prepared eluates from the red cells of two patients were nonreactive. A possible explanation is that the heat elution technique did not provide optimum conditions for the dissociation of INOX-immunoglobulin complexes from the patients' red cells or for the readsorption of those complexes onto red cells reacted with the eluate.

The findings in these experiments correspond with the behavior of Schiff's bases which are pH dependent, and easily hydrolyzed by acids.[9] The attachment of INOX to red blood cells is corroborated by the uptake of radiolabeled INOX-8-C14 by red cells using techniques similar to those reported by Kimball et al (Fig 2).[10]

INOX most likely acts to produce a positive DAT in vivo in a manner similar to the proposed in vitro mechanism, ie, by the condensation of an aldehyde group with amino acids of serum proteins and red cell membrane proteins to form Schiff's bases. Although immunoglobulin can be detected on the red cells of patients who have received intravenous infusions of INOX, the sensitization of these red cells does not appear to result in clinical evidence of hemolysis. This might be explained by the data that suggest that INOX results in the covalent attachment of immunoglobulin to red cells via the formation of Schiff's bases and not due to the specific interaction between the Fab portion of immunoglobulin molecules and red blood cells. The failure of immunoglobulin molecules to bind to red cells via their Fab portion could result in the inaccessability of the Fc portion of the immunoglobulin molecules to the Fc receptors present on macrophages, thus preventing both erythrocyte-macrophage interaction as well as hemolysis of the sensitized red cells.

INOX has not been used in phase I or II trials for five or six years and is not likely to be used widely in the future due to hepatic toxicity and relative ineffectiveness. However, these findings may be of potential practical importance since they offer a new explanation of how drugs can induce a positive direct antiglobulin test. In theory, any drug or chemical containing two or more aldehyde or acid groups capable of reacting with proteins under physiologic conditions could produce a positive direct antiglobulin test. Drugs or chemicals in this category include Evans blue, folic acid, maleic acid, glutaric acid, glyceryl trinitrate (nitroglycerin), menadiol sodium phosphate (synkavite), penillic acid, phthalic acid, picric acid, sodium iodipamide (cholografin sodium), and sodium iodomethamate (iodoxyl). Studies of these and other agents are being planned or conducted by our group.

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Synchronized Cultures of *P falciparum* in Abnormal Red Cells: The Mechanism of the Inhibition of Growth in HbCC Cells

By Julie A. Olson and Ronald L. Nagel

It has been previously demonstrated that HbCC cells fail to support growth of *P falciparum* in asynchronous Jensen-Trager cultures. To define the mechanism of inhibition we have studied synchronous cultures and found that while intraerythrocytic parasite development appeared normal, the liberation of merozoites and/or invasion was impaired. This effect was detected by a normal growth during the first growth cycle but dramatically reduced number of ring forms following the schizont stage. A specific test for the invasion of CC cells by *P falciparum* merozoites, nevertheless, was normal. The defect found in infected CC cells was not modified by changes in O_2 tension (which altered the ligand saturation of Hb C) nor by the extracellular K^+ concentration (excluding a K^+ leak-dependent mechanism for the growth inhibition). The osmotic lysis of late-staged parasitized red cells revealed that 25% of infected AA cells were lysed when the extracellular medium was 95 mOsm. In contrast, infected CC cells required a decrease to 10 mOsm in the extracellular media to reach 25% lysis. We conclude that CC red cells are unsuitable hosts for the malarial parasite primarily because of their inability to lyse and release merozoites at the appropriate stage of intraerythrocytic development of *P falciparum*.

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IT HAS long been suspected that genetically abnormal human red cells found in individuals living in malaria endemic regions offer their carriers protection against mortality and morbidity from *P falciparum* infections.[1,2] Specifically HbC, the distribution of which is restricted to malaric areas in and around Upper Volta (Burkina-Faso) has been considered a malaria-selected red cell mutation although there is conflicting evidence.[3,4] In the last 5 years considerable understanding of the mechanisms involved in genetically determined cellular protection have been derived[5-8] from studies in cultures of the type described by Jensen and Trager.[9]

The objective of the present study was to determine the basis of the growth inhibition of *P falciparum* observed in red cells of individuals homozygous for HbC.[7] There are several stages in the erythrocytic cycle at which parasite infection could be inhibited. The first would be erythrocyte invasion by merozoites; several red cell membrane variants have been described that are refractory to merozoite invasion.[2,8] The next stage susceptible to inhibition is the intracellular parasite development. This category would include impairments in the parasite's ability to meet its nutritional requirements or changes in the host cell milieu that would be cidal to the parasite. G6PD deficiency and sickle cell anemia and trait, fall into this group.[10,11] Finally, impairment of red cell rupture and release of merozoites at parasite maturity could inhibit infection. No instances of this abnormality have yet been described.

We report here the invasion, effect of O_2, and extracellular K^+ level, and the consequence of the presence of the parasite on the osmotic response of HbC-containing cells, using synchronized in vitro cultures that mimic the growth pattern in the human infection. The data obtained strongly suggest that the mechanism involved in the HbC-containing cells is the impairment of the parasite's ability to produce cell lysis and the consequent unavailability of merozoites for further red cell infection.

MATERIALS AND METHODS

Cultivation of P falciparum. The FCR3 strain of *P falciparum* was maintained in culture[9] in synchronous growth[12] at 5% cell suspension, with daily replacement of medium.

Culture media. Unless otherwise stated, parasites were grown in RPMI 1640 (GIBCO, Grand Island, NY), 25 mmol/L HEPES (Sigma, St. Louis), 40 ug/mL gentamycin (Schering Pharmaceutical Corp, Kenilworth, NJ), and 10% (v/v) defibrinated human plasma (Jacobi Hospital Blood Bank, Bronx, NY). Such medium is referred to as RPS.

High potassium (HK) and low potassium (LK) media were prepared as described by Friedman et al.[11] In brief, all the components except the salts of the Selectamine RPMI 1640 kit (GIBCO) were combined. The medium was brought to 0.5 mmol/L $CaCl_2$, 0.4 mmol/L $MgCl_2$, 5.6 mmol/L Na_2HPO_4, and 0.2 g/L $NaHCO_3$. For HK, KCl was added to 105 mmol/L. LK was adjusted to 100.2 mmol/L NaCl and 5.4 mmol/L KCl. HEPES, gentamycin, and serum were added as for RPS.

Assessment of invasion. The susceptibility to merozoite invasion was assessed as described by Olson and Kilejian.[13] In 11 separate cultures (performed in triplicate) from 5 different HbCC donors, the proportion of invaded red cells was compared to invasion of HbAA cells in a parallel culture receiving the same inoculum.

Assessment of growth. Blood samples were collected into CPD and used within 2 weeks of collection. Hemoglobin composition was determined by cellulose strip electrophoresis (pH 8.6) and by Corning agar electrophoresis (pH 6.4). Just prior to infection, blood was washed in RPS without serum to remove the plasma and buffy coat. Cells were counted in a hemacytometer from duplicate dilutions and adjusted to 5×10^8 RBCs/mL (approximately a 1% cell suspension) in RPS. Inoculating parasites were obtained by gelatin enrichment[14] from schizont-containing cultures to greater than 60% parasitemia. Schizont-infected RBCs were added to test RBCs to achieve a final parasitemia of 1% (5×10^6 schizonts/mL). Samples were plated in 24 well Linbro plates (0.5 mL/well) and incubated under candle jar conditions unless otherwise indicated. Cultures grown in "3% O_2" were incubated in a gas mixture 3% O_2, 3% CO_2, 94% N_2, and at "17% O_2," in a mixture of 17% O_2, 3% CO_2, 80% N_2.

After 24 hours of growth (ie, through the first invasion), the culture medium was changed, either to HK, LK, or RPS, as indicated. Thereafter, medium was changed daily. Every 24 hours after infection, from each sample, 3 wells were collected to each of

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three 12 × 75 mm tubes, centrifuged, and adjusted to approximately 50% cell suspension by removal of the medium. The suspensions were mixed, smeared, fixed in methanol, stained in Giemsa, and the % parasitemia determined by counting 1000 cells from each of the three smears for each sample Percentage of parasitemia is expressed as the average of the three determinations ± SEM.

Red cell volume determinations. Red cell volume (MCV) was calculated from triplicate cell counts in a hemacytometer and hematocrit (International, Microcapillary centrifuge) as follows. 1/(cells/mL − % hematocrit).

Osmotic sensitivity determinations Washed RBCs from HbAA or HbCC donors were inoculated with gelatin-enriched schizont-infected RBCs to 5% parasitemia and plated at a 1% cell suspension. After 42 hours incubation under candle jar conditions (with a change of medium at 24 hours), cells were collected, centrifuged at 1000 × g for 5 minutes, the supernatant medium removed, and the cell pellet mixed on a vortex mixer. Twenty-five μL of cell pellet was transferred to the bottom of a 12 × 75 mm tube. Two volumes (50 μL) of hypoosmotic medium (see below) were added while the tube was being vortexed and the sample was immediately drawn into a Pasteur pipette, thin smeared, and dried under a stream of air Thin smears were fixed in methanol and stained in Giemsa. The % of lysed, infected RBCs was calculated by dividing the number of lysed, infected RBCs (schizonts and clusters of merozoites not enclosed within RBC membrane and schizonts within RBC membranes within which the pinkish, hemoglobin stain was not visible) by the total number of infected RBCs (lysed plus unlysed, ie, schizonts within RBC membranes within which hemoglobin staining was observed) Hypoosmotic media were prepared by diluting Dulbecco's PBS (290 mOsm) with water to the desired osmolarity.

RESULTS

When AA and heterozygous AC RBCs were compared under standard culture conditions (Fig 1), there was no significant difference in parasite growth between the two cell types. However, parasite growth was markedly inhibited in homozygous CC RBCs (Fig 1). Invasion of HbCC cells was 85.0% ± 14.8% SD (n = 11) of control invasion of HbAA cells. Parasite morphology in HbCC cells appeared normal on day 2. Inhibition of growth in HbCC cells was only apparent in the second cycle of development (days 3 and 4) and was manifested as a lack of increase of parasitemia. It was observed that, in the CC cultures depicted in Figure 1A, there were many degenerated schizonts and segmenters on day 3.

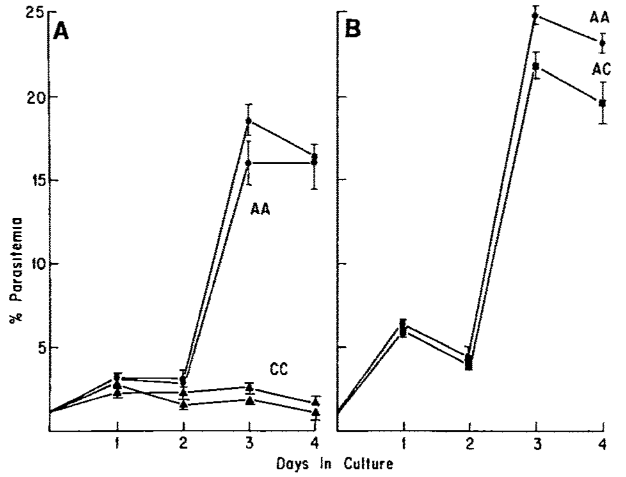

Fig 1. Synchronous growth of *P falciparum* in HbAA, HbAC, and HbCC erythrocytes at 17% O_2. Growth was assessed over four days (see Methods) in (A) 2 HbAA donors (closed circles) and 2 HbCC donors (closed triangles), and (B) a HbAA donor (closed circles) and a HbAC donor (closed squares).

To determine whether growth inhibition in CC RBCs resembles that in AS or SS RBCs,[11] growth was compared in high and low potassium media. Parasite growth in AA cells was consistently inhibited in high potassium medium (Fig 2, open *v* closed circles). Cultivation in the presence of high potassium medium did not reverse the inhibitory nature of CC cells (Fig 2, open *v* closed triangles); in fact, parasite growth was even further impaired in high potassium. MCV determinations indicated that incubation in high potassium medium did not alter red cell volume relative to that in low potassium medium in either AA or CC cells.

Cultivation of *P falciparum* in CC RBCs under conditions of low oxygen tension (3% oxygen) did not decrease the parasitemia, as is the case in AS and SS cells.[2] In fact, the parasitemia was slightly increased (Fig 3) in both AA and CC cells at low oxygen. Incubation in high potassium medium at low oxygen did not alter the pattern of growth in CC cells (data not shown).

The effects of changes in potassium content of the medium and oxygen tension during cultivation indicated that the mechanism of growth inhibition in CC cells did not resemble that in SS or AS cells.[11] However, since studies of *P falciparum* growth in AS and SS cells had been carried out

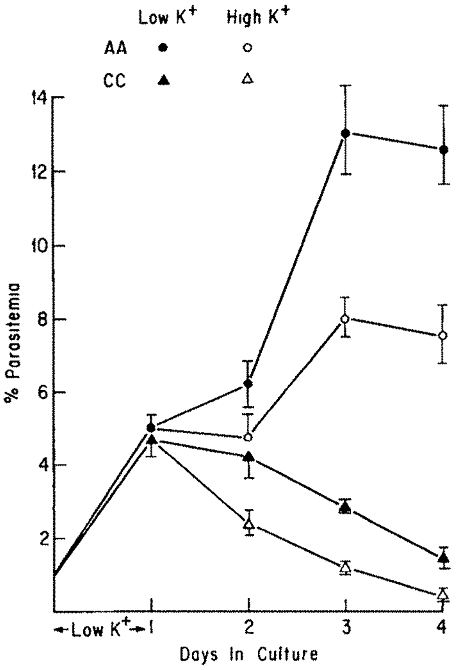

Fig 2. Synchronous growth of *P falciparum* in HbAA and HbCC erythrocytes in high and low potassium medium at 17% O_2. After merozoite invasion in low potassium medium (days 0 to 1), cells were transferred to high potassium medium (HbAA open circles, HbCC open triangles) or low potassium medium (HbAA closed circles, HbCC closed triangles) and growth was assessed for the following 3 days.

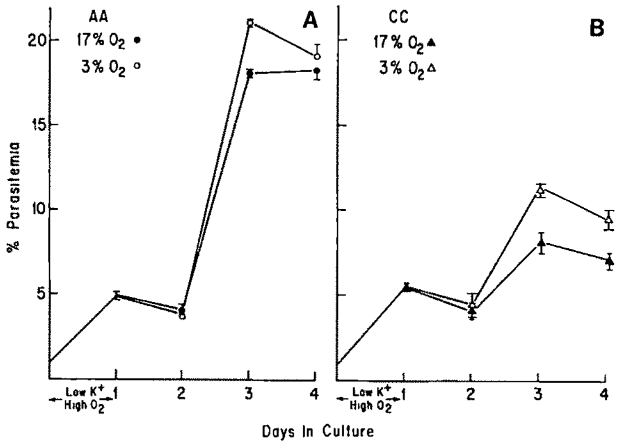

Fig 3. Synchronous growth of *P falciparum* in (A) HbAA and (B) HbCC erythrocytes at high (17%) and low (3%) oxygen tension in low potassium medium. After merozoite invasion at 17% O_2 (day 0 to day 1), cultures were transferred either to 17% O_2 (HbAA closed circles, HbCC closed triangles) or 3% O_2 (HbAA open circles, HbCC open triangles) and growth was assessed for the following 3 days.

in asynchronous cultures, it was deemed important to repeat these experiments under conditions of synchronous growth and to follow the complete course of growth over 4 days. Fig 4 depicts the results of experiments in which parasite growth over a four-day period was assessed in AA (Fig 4A), SS (Fig 4B), and AS (Fig 4C) cells under conditions of high and low potassium and high and low oxygen. As noted above (Fig 2), parasite growth was inhibited in AA cells in high potassium, both at high and low oxygen (Fig 4A). In addition, growth was increased at low oxygen.[15] Unlike parasite growth in CC cells, growth was almost normal in both AS and SS cells at high oxygen (Fig 4B and 4C, closed circles). In both AS and SS cells, growth was reduced in the presence of high potassium media at high oxygen (Figure 4B and 4C, closed triangles). At low oxygen tension, parasite growth in SS cells was severely inhibited (Figure 4B, open circles) and the inhibition was not reverse by cultivation in the presence of high potassium medium (Figure 4B, open triangles). However, in AS cells, the inhibition of growth at low oxygen (Figure 4C, open circles) was reversed by incubation in high potassium medium (Figure 4C, open triangles). This reversal was most apparent on day 3.

The observation that segmenters remained in the CC culture on day 3 (Figure 1A), taken in conjunction with the known osmotic resistance of CC cells,[16] led us to postulate that parasites that had developed in CC cells were not able to overcome the osmotic barrier, and hence could not release merozoites at the appropriate time. Osmotic resistance in *Plasmodium*-infected CC cells has not been studied. The experiment illustrated in Fig 5 was designed to observe osmotic sensitivity of infected cells in a mixed population of infected and uninfected cells. The L25 (osmolarity of added media at which 25% of the infected cells were lysed) for AA cells was approximately 95 mOsm and for CC cells was 10 mOsm.

DISCUSSION

There are several properties of HbC-containing cells that could protect against *P falciparum* infection. First, the cells have a decreased deformability. In some cases, cells with reduced deformability are refractory to invasion.[17,18] Second, CC cells are hyperdense and can even contain intracellular crystals of hemoglobin.[19] Such properties could impair the parasite's ability to develop within the cells, in much the same way that *P falciparum* growth is inhibited in HbS polymer–containing cells.[6] In addition, the reduction in cation content (Na^+, K^+),[20] could have a deleterious effect on parasite development. Third, CC cells are osmotically resistant,[16] and might trap the parasite by inhibiting host cell rupture.

The reduced deformability and suspected membrane changes in HbCC cells[21] do not appear to interfere with merozoite invasion, as demonstrated here. Furthermore, development of the parasite through schizogony appears to be identical to that in HbAA cells under all conditions tested here. The possibility that slight inhibition of intracellular parasite development also makes a minor contribution to the abnormal growth pattern in CC cells cannot be rigorously excluded without additional studies demonstrating normal metabolic junctions (hypoxanthine incorporation, amino acid incorporation, glucose utilization, etc) during schizogony. Cytosol phase conditions (ie, presence or absence of aggregated hemoglobin C) and potassium concentration in HbCC cells do not seem to influence intracellular development, in contrast to the findings in HbSS and HbAS erythrocytes restudied here (Fig 4).[11] We have reexamined the growth curve of *P falciparum* in AA and AS cells in

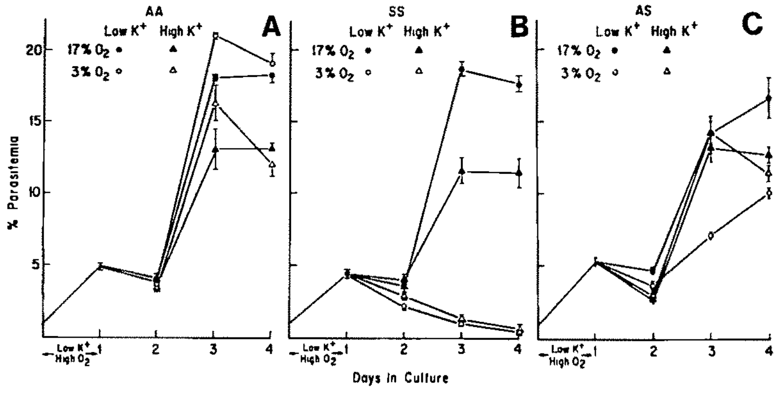

Fig 4. Synchronous growth of *P falciparum* in HbAA, HbAS, and HbSS erythrocytes at high and low O_2, and in high and low potassium medium. After merozoite invasion in low potassium medium at 17% O_2, cultures were transferred to high or low potassium medium and high or low O_2. (A) HbAA, (B) HbSS, and (C) HbAS. Closed circles—low potassium and 17% O_2, open circles—low potassium and 3% O_2, closed triangles—high potassium and 17% O_2, open triangles—high potassium and 3% O_2.

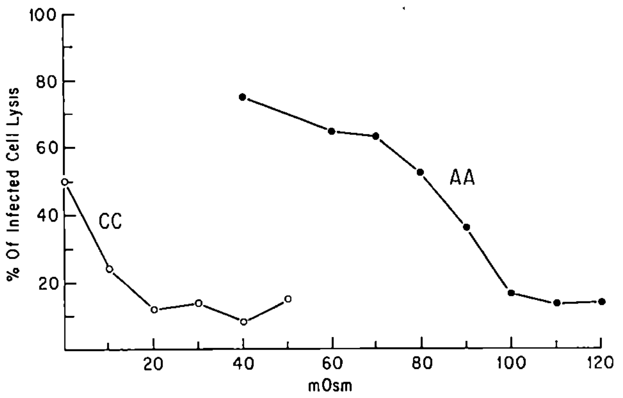

Fig 5. Osmotic sensitivity of *P falciparum* schizont-infected HbAA and HbCC erythrocytes. Osmotic lysis of infected HbAA (closed circles) and HbCC (open circles) was assessed as described in the Methods. The abscissa shows the osmolarity of buffer added to infected cell pellets.

synchronized cultures and have confirmed results obtained from asynchronous cultures.[11] Parasite growth in SS and AS cells is only impaired under conditions of low oxygen tension. At low oxygen and at the lowered intracellular pH of infected SS cells, HbS polymerizes and the parasite is probably killed by an inability to use this polymerized hemoglobin as a food source[22] and mechanical rupture of the parasite by HbS polymers.[23]

In AS cells, parasite growth is inhibited at low O_2 and normal growth is restored in a high K^+ medium. The mechanism of the growth inhibition in AS cells could be an intrinsic sensitivity of the parasites to a low K^+ environment or that K^+ leak-induced dehydration (coupled with increased intracellular pH) could increase intracellular polymer formation, which in turn, as in the case of SS cells, might not be a good substrate for the parasite proteases.[22] The high K^+ medium then, by inhibiting K^+ egress, restores normal growth.

We turn now to CC cells. First, unlike AS and SS cells, parasite growth was inhibited at high oxygen. In fact, at low oxygen, CC cell growth was slightly enhanced, as in AA cells. Second, incubation in high potassium did not restore normal growth. In fact, high K^+ (105 mmol/L) was inhibitory relative to normal low K^+ (5.4 mmol/L) medium, both at high oxygen and at low oxygen. We can conclude from these observations that the mechanism of inhibition of *P falciparum* growth in CC cells does not involve loss of K^+ and subsequent dehydration.

The observation that many degenerating schizonts are detected on day 3 in the CC cultures (and even on day 2), combined with the observed high osmotic resistance of parasitized CC cells (Fig 5), suggest that these abnormal cells represent too great a barrier for the parasite to overcome in the process of host-cell rupture. The mechanism of parasite-induced red cell lysis is probably a combination of well-known increases in host cell membrane permeability during parasite development[24] and the possible participation of parasite proteases and lipases that attack the membrane. The role in parasite growth inhibition played by the membrane transport abnormalities recently reported in CC cells[25] are yet to be determined.

The normal parasite growth in AC cells can be readily understood, in the context discussed, by the fact that they exhibit a much less abnormal osmotic lysis curve than CC cells, particularly between 95% and 100% lysis. At this range, AC cells are lysed at the same osmotic environment as AA cells.[16]

It is appropriate to point out that although AC cells appear to sustain normally the growth of *P falciparum,* this does not exclude the possibility that these cells could offer the carriers fitness advantages by other mechanisms. For example, AC individuals might have retardation of the switch from Hb F to A in the critical first years of life. As Hb F–containing cells do not sustain parasite growth normally,[26] the carrier can be rendered significantly resistant to the disease. In addition, in vitro growth experiments do not model all of the pathologic events in vivo. For example, it is possible that infected AC cells have abnormal membrane knobs that might alter knob-mediated deep vascular schizogony,[27] although data are not available to prove the advantage of AC individuals in malaric regions. These are difficult experiments, and a final conclusion should wait for further investigation.

We conclude that while AC cells sustained normally the growth of *P falciparum* in vitro, CC cells do not allow normal development of the parasite, primarily because the abnormal osmotic response of parasitized CC cells interferes with red cell rupture needed for the liberation of infective merozoites.

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Purified Interleukin-3 and Erythropoietin Support the Terminal Differentiation of Hemopoietic Progenitors in Serum-Free Culture

By Junko Suda, Toshio Suda, Kazuo Kubota, James N. Ihle, Masaki Saito, and Yasusada Miura

We studied the effect of purified interleukin-3 (IL-3) and erythropoietin on colony formation by hemopoietic progenitors in serum-free cultures of spleen cells from 5-fluorouracil (5-FU)-treated mice. In the presence of IL-3 alone, most of the multilineage (three or more lineages) colonies did not contain erythroid cells. However, in the presence of IL-3 and erythropoietin, most of the multilineage colonies contained various numbers of erythroid cells. Replating experiments suggest that IL-3 maintains the growth of the progenitor cells, which could differentiate into erythroid cells. Erythropoietin facilitated the terminal differentiation and amplification of erythroid cells, although it did not sustain the growth of multipotential stem cells. Single-cell transfer experiments demonstrate that IL-3 supported the late stages of differentiation of neutrophils, macrophages, eosinophils, and megakaryocytes in the absence of lineage-specific factors. Therefore, IL-3 supports the differentiation of multilineage hemopoietic progenitors, and the terminal differentiation of most hemopoietic lineages, with the exception of the erythroid lineage.

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ONE important question in hemopoiesis is how multipotential hemopoietic progenitors develop into the different blood cells. Clonal growth of hemopoietic cells requires hemopoietic factors. Highly purified fractions of WEHI-3–conditioned medium have been shown to support the growth and differentiation of cells of several blood cell lineages.[1,2] The protein for this activity has been purified to apparent homogeneity and was termed interleukin-3 (IL-3).[3] IL-3 has been demonstrated to have mast cell growth factor activity and granulocyte-macrophage colony-stimulating activity.[4] Another hemopoietic factor, human erythropoietin, has been isolated from urine of aplastic anemic patients in a high yield with a simple purification procedure using an immunoabsorbent column of monoclonal antibodies.[5,6]

On the other hand, a highly enriched population of hemopoietic progenitors has been available. Nakahata and Ogawa identified a new class of murine hemopoietic colonies which consist of undifferentiated blast cells that had a high secondary replating efficiency in culture.[7] Subsequently, based on the observation by Bradley and Hodgson,[8] we observed that 5-fluorouracil (5-FU) treatment of mice provided significant enrichment of the progenitors.[9] Recently, we showed that purified IL-3 supported the formation of both blast cell colonies and multilineage colonies.[10]

The in vitro systems used in the above experiments contain substantial amounts of sera which may have erythropoietin and other hemopoietic factors. Guilbert and Iscove initially demonstrated that serum could be replaced by a chemically defined media for the growth of murine hemopoietic progenitors.[11,12] We reported a modification of this method for the growth of granulocyte-macrophage colonies using agar cultures.[13] More recently, the growth of primitive erythroid and nonerythroid progenitors in serum-free medium has been described.[14,15]

We studied the effects of highly purified IL-3 and erythropoietin on the differentiation and proliferation of hemopoietic progenitors in a serum-free methylcellulose culture system. We also examined the role of IL-3 in the terminal differentiation of hemopoietic progenitors in single-cell transfer experiments.

MATERIALS AND METHODS

Cell preparation. Ten- to 15-week-old female BDF$_1$ mice were obtained from the Shizuoka Experimental Animal Center (Shizuoka, Japan). We administered 5-FU (F Hoffmann-La Roche & Co, Ltd Basel, Switzerland) at a dosage of 150 mg/kg of body weight through the tail vein[8,9] Spleen cells were harvested 4 days after the 5-FU injection and single-cell suspensions were prepared from pooled spleens of three mice by washing them three times with Iscove's modified Dulbecco's tissue culture medium (IMDM; GIBCO Laboratories, Grand Island, NY).

Serum-free media. The serum-free media were prepared by a modified version of the method of Iscove et al[11,13] One mL of 1.2% methylcellulose (Fisher Scientific, Norcross, Ga) in IMDM contained 15 mg of bovine serum albumin (BSA; Sigma Chemical, St. Louis, Mo), 600 μg of human transferrin (Sigma Chemical), 160 μg of L-χ-phosphatidylcholine (Sigma Chemical), 96 μg of cholesterol (Wako Pure Chemical Industries, Osaka, Japan), and 400 U of IL-3 in the presence or absence of 2 U of highly purified human urinary erythropoietin (Snow Brand Milk Products Co, Ishibashi, Japan).

BSA was deionized with ion exchange resin AG 501-X8(D) (Bio-Rad Laboratories, Richmond, Calif) according to the method of Tepperman et al[16] and an isotonic solution was made with IMDM BSA used in this study was essentially fatty acid free and globulin free. Before use, 0.1 mL of 7.5% sodium bicarbonate was added to each 2.5 mL of BSA and the pH adjusted to 7.2. Transferrin was fully iron-saturated with FeCl$_3$. Cholesterol and L-α-phosphatidylcholine were dissolved in IMDM according to the method of Iscove et al.[11,12] Before use, 7% sodium bicarbonate was added in the same manner as BSA. IL-3 was purified to homogeneity from WEHI-3–conditioned medium.[3] We used purified IL-3 with a specific activity of 5 × 10^6 U/mg protein. The stock solution contained 40,000 U/mL of activity and was kept frozen in PBS with 1 mg/mL BSA. Erythropoietin purified from human urine using monoclonal antibody technique was generously provided by Dr Masatsugu Ueda in Snow Brand Milk Products Co. It had a specific activity of 81,600 U/mg protein.[5]

Clonal cell culture. Cultures of 1.2 × 10^6/mL spleen cells from 5-FU–treated mice were prepared in 35 mm nontissue culture dishes (Falcon, Oxnard, Calif) of serum-free methylcellulose medium

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containing 400 U/mL of IL-3 and/or 2 U/mL of erythropoietin or 10 μg/mL of BSA as a control. Colony growth was observed daily under an inverted microscope. When colonies appeared to have matured, individual colonies were lifted with a 3 μL Eppendorf micropipette and cell number was counted using a hemocytometer. The samples were spun in a cytocentrifuge (Cytospin, Shandon Southern, Sewickley, Pa) and stained with May-Grunwald-Giemsa staining as described previously.[17] In order to confirm lineage, we used specific staining; dual esterase staining for granulocytes and macrophages,[18] toluidine blue staining for mast cells, benzidine staining for erythroid cells, and cholinesterase staining for megakaryocytes.[19]

Replating experiment of blast cell colonies. On day 10 of cultures containing only IL-3, blast cell colonies were picked up from the methylcellulose medium and suspended in 0.1 mL of IMDM. The sample was added with 400 U/mL IL-3 and 2 U/mL erythropoietin in the culture dish. The dish was gently agitated in order to disperse the cells. Cultures of replated cells were then incubated at 37 °C in 5% CO_2.[9] The multilineage colonies were lifted up on day 12 of incubation and examined for cell number and the morphology of the individual colonies.

Single cell transfer experiments. Spleen cells from 5-FU–treated mice were cultured in serum-free methylcellulose medium in the presence of 400 U/mL of IL-3. Blast cell colonies consisting of 50 to 100 cells were picked up as mentioned above on days 10 to 14 of culture. The cells were suspended in 1 mL of serum-free methylcellulose medium containing 400 U/mL of IL-3 in the second 35-mm culture dish. Transfer of a single cell to the third culture dish containing 400 U/mL of IL-3 in 1 mL serum-free methylcellulose medium was performed under direct microscopic visualization using a fine Pasteur pipette with a diameter of approximately 30 μm which was attached to a micromanipulator (Narishige Scientific Instrument Lab, Tokyo, Japan). After confirmation of the presence of a single cell in each dish, incubation was carried out. On 6 to 10 days of incubation, when the colonies appeared to have matured, individual colonies were lifted. Samples were spun in Cytospin and stained with May-Grunwald-Giemsa.[20,21]

RESULTS

Effect of IL-3 and erythropoietin on colony formation. Blast cell colonies and multilineage colonies were formed in the serum-free methylcellulose medium in the presence of purified IL-3. The number of colonies formed in this culture was about half that formed in the serum-containing culture. In the serum-free culture, blast cell colonies appeared later than in the serum-containing culture system.[10] As a control, 10 μg/mL BSA, which was used in the IL-3 stock preparations, did not support the colony formation, nor did erythropoietin support the colony formation.

The numbers and types of colonies supported by IL-3 with or without erythropoietin were compared. On days 14 to 21 of incubation, when the colonies appeared to have matured, individual colonies were lifted from the methylcellulose medium and examined morphologically. Addition of 2 U/mL of highly purified erythropoietin caused a significant change in the appearance of erythroid lineage in the colonies. Fig 1 shows representative multilineage colonies and cytospun preparation of both groups. Table 1 shows the numbers of various types of colonies identified from three dishes. In the presence of IL-3 and erythropoietin, 33 out of 55 colonies were multilineage (three or more lineages) colonies, 31 of which contained erythroid lineage. By contrast, in the presence of IL-3 alone, 29 out of 56 colonies were multilineage, 26 of which did not contain erythroid lineage. The numbers of neutrophil-macrophage colonies and macrophage colonies in both groups were similar. On day 21 of incubation, 9 blast cell colonies were still observed in the dishes containing IL-3 and erythropoietin, and 2 blast cell colonies remained in the dishes containing IL-3 alone. Each blast cell colony consisted of 20 to 100 cells, which showed no sign of differentiation.

There were no significant differences in the percentage of megakaryocyte, eosinophil, neutrophil, macrophage, or blast cell in the multilineage colonies in each dish (Table 2). Fig 2 shows a comparison of percentage of erythroid cells in multilineage colonies in IL-3 plus erythropoietin and IL-3 alone. In the presence of IL-3 and erythropoietin, 31 out of 33 multilineage colonies contained erythroid cells, ranging from 2.0% to 93.6% of the total cells per colony. On the other hand, in the presence of IL-3 alone, only 3 out of 29 multilineage colonies contained erythroid cells which were less than 5% of the total cells per colony. These cells were benzidine positive and some of them denucleated. Although the size of the multilineage colonies varied widely, the cell number in each colony was significantly correlated with the

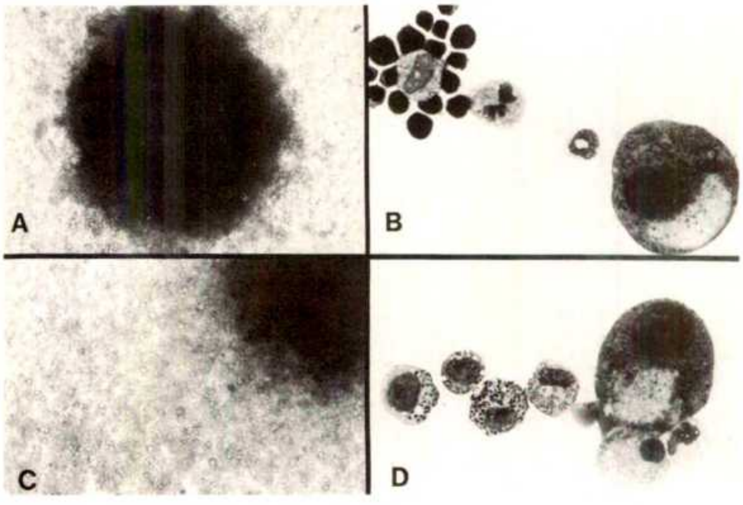

Fig 1. (A) A representative multilineage colony seen on day 14 of the serum-free culture in the presence of IL-3 and erythropoietin. This colony shows red color and a variable size of cells. (B) A portion of a May-Grunwald-Giemsa stained smear of the multilineage colony shown in (A), revealing erythroblasts, a neutrophil, and a megakaryocyte. (C) A representative multilineage colony seen on day 14 of the serum-free culture in the presence of IL-3 alone. Color was not red. (D) A portion of a May-Grunwald-Giemsa stained smear of the multilineage colony in (C), revealing a neutrophil, a macrophage, mast cells, and a megakaryocyte.

Table 1. Number of Various Types of Colonies Formed by IL-3 in the Presence or Absence of Erythropoietin

Lineage							
E	M	e	mast	n	m	IL-3 + Ep*	IL-3*
+	+	+	+	+	+	5	1
+	+	+	+		+	1	0
+	+		+	+	+	17	1
+	+		+	+		0	1
+	+		+		+	1	0
+	+			+	+	7	0
	+	+	+	+	+	0	3
	+	+		+	+	1	1
	+		+	+	+	0	15
	+			+	+	0	2
	+					0	2
		+	+	+	+	0	2
		+		+	+	1	1
			+	+	+	0	2
			+	+		0	1
				+	+	5	6
				+		0	1
					+	17	17
Totals						55	56

Abbrev: Ep, erythropoietin; E, erythrocyte; M, megakaryocyte; e, eosinophil; mast, mast cell; n, neutrophil; m, monocyte-macrophage.

*Pooled data from three dishes containing 1.2 × 10^6 cells/mL.

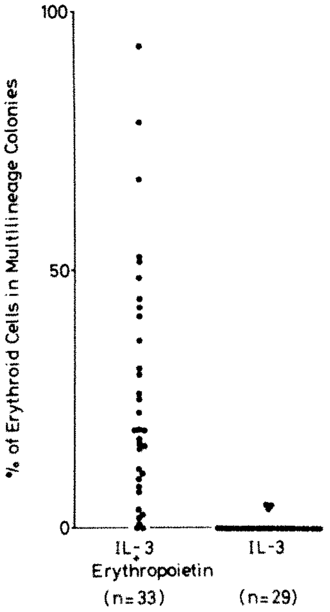

Fig 2. Comparison of percentage of erythroid cells in multilineage (three or more lineages) colonies supported by IL-3 + erythropoietin and IL-3 alone. Each point shows an individual multilineage colony.

percentage of erythroid cells in each colony ($r = 0.55$, $P < 0.01$).

Replating experiment of blast cells. Blast cells in the medium containing IL-3 alone were replated into medium containing IL-3 and erythropoietin. A total of 320 blast cells formed 184 colonies, 9 of which were multilineage colonies. Seven of these colonies contained erythroid lineage; 5 EMmastm colonies and 2 EMnm colonies.

Colony formation of single progenitors. The single cells from blast cell colonies grown in the presence of IL-3 were transferred to new serum-free culture medium containing 400 U/mL IL-3. Fig 3 shows an undifferentiated cell from

Table 2. Size and Composition of the Multilineage Colonies (Three or More Lineages) Formed by IL-3 in the Presence or Absence of Erythropoietin

Colony Number	Day of Analysis	No of Cells per Colony	Differential Count* (%)							
			E	M	e	mast	n	m	Bl	Lineage
IL-3 + Ep										
1	14	6.6 × 10^4	43.2	3.2	1.2	0.4	10.4	40.8	0.8	EMemastnm
2	16	3.8 × 10^4	8.8	4.4		0.8	63.0	23.0		EMmastnm
3	16	14.5 × 10^4	52.0	4.0			22.4	19.2	2.4	EMnm
4	16	7.2 × 10^4	16.8	1.6			24.8	48.8	8.0	EMnm
5	21	25.9 × 10^4	79.2	1.2		3.6	0.8	2.8	12.4	EMmastnm
6	21	1.0 × 10^4	52.8	7.2		8.8	3.2	20.8	7.2	EMmastnm
7	21	3.5 × 10^4	3.6	0.4		0.4	68.8	4.4	22.4	EMmastnm
8	21	0.5 × 10^4	17.6	1.6			60.0	12.8	8.0	EMnm
IL-3										
1	16	0.8 × 10^4		2.4	3.2		32.8	61.6		Menm
2	16	5.0 × 10^4		0.8		2.4	33.6	58.4	4.8	Mmastnm
3	16	8.2 × 10^4		4.0		7.2	66.4	15.2	7.2	Mmastnm
4	16	7.7 × 10^4		1.6			72.0	20.8	5.6	Mnm
5	16	3.1 × 10^4		0.8			12.8	86.4		Mnm
6	16	7.4 × 10^4			0.8	0.8	8.8	89.6		emastnm
7	20	0.9 × 10^4	4.8	4.8		7.2	43.2	8.0	32.0	EMmastnm
8	20	1.0 × 10^4		1.6		1.6	7.2	88.0	1.6	Mmastnm
9	20	4.1 × 10^4		1.6		45.6	44.8	5.6	2.4	Mmastnm
10	20	1.3 × 10^4			1.6		1.6	96.8		enm

Abbreviations same as in Table 1.

*Differential counting was carried out on 250 cells.

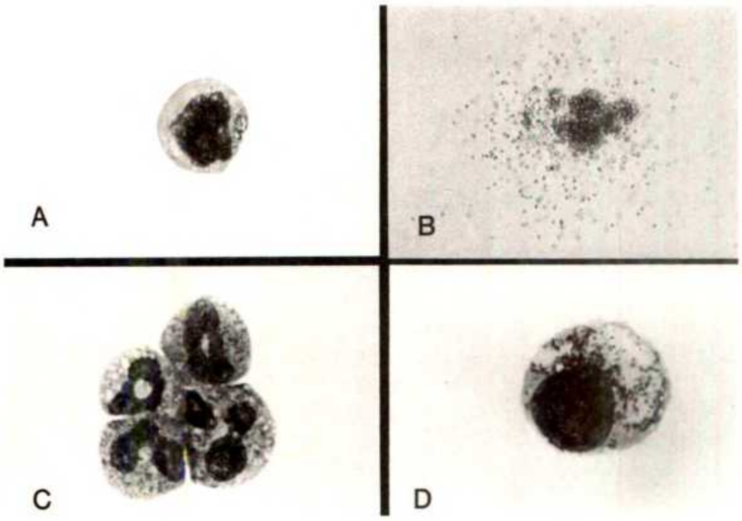

Fig 3. (A) An undifferentiated cell from blast cell colonies, used for single cell transfer. (B) A neutrophil-macrophage colony derived from a single progenitor in the serum-free culture containing IL-3 alone. (C) Neutrophils in the colony shown in (B). (D) A megakaryocyte derived from a single progenitor.

blast colonies, which served for single cell transfer. Of a total of 208 single cells, 111 yielded colonies. Of these, 62 colonies were picked up and the morphology examined. There were 56 macrophage colonies, 2 megakaryocyte colonies, 1 eosinophil colony, and 3 neutrophil-macrophage colonies. Fig 3 shows a neutrophil-macrophage colony derived from a single cell in the serum-free culture dish containing IL-3. It also presents the mature neutrophils and a megakaryocyte supported by IL-3.

DISCUSSION

Hodgson and Bradley demonstrated that high concentrations of developmentally early cells were obtained by treating mice with 5-FU.[8] Following the injection of 5-FU, the numbers of erythroid progenitors and granulocyte-macrophage progenitors are markedly reduced. On the contrary, the numbers of multipotential hemopoietic cells are less affected by this treatment.[9] Using spleen cells from 5-FU–treated mice, we have shown that IL-3 supports the blast cell colony formation and multilineage colony formation. In a serum-containing culture system, however, the addition of 2 U/mL of erythropoietin did not cause any significant change in the number of multilineage colonies containing erythroid cells.[10]

In this study, we showed that IL-3 supports the formation of blast cell colonies and multilineage colonies in serum-free cultures. In IL-3 alone, a few multilineage colonies contained a low percentage of erythroid cells. The low frequency of erythroid cells may be due to the ability of IL-3 to support the terminal differentiation of erythroid cells. Alternatively, the bovine serum albumin and human transferrin used in this study might contain a small amount of erythropoietin. However, a significant level of erythropoietin was not detected by a radioimmunoassay. Values in the range of 10 mU/mL are near the lower limit of sensitivity of this assay. Last, cells in multilineage colonies or scattered cells in the culture dish might produce erythropoietin or erythropoietin-like substance(s), but no erythropoietin was detected in the media conditioned by spleen cells. Most recently, Goodman et al reported that IL-3 stimulates the full development of many erythroid burst formations in serum-free cultures without detectable erythropoietin.[23] The cause of the difference is unknown. Contaminants other than erythropoietin in the bovine serum albumin might contribute to the apparent erythropoietin dependence.

In the presence of IL-3 and erythropoietin, most of the multilineage colonies contained erythroid cells. The replating experiments of blast cells from the dishes containing IL-3 to the dishes containing IL-3 and erythropoietin suggest that IL-3 maintains the growth of progenitor cells which could differentiate to erythroid cells. On the other hand, erythropoietin did not support any kind of colony formation of spleen cells from 5-FU–treated mice. From these findings, we concluded that erythropoietin facilitates the terminal differentiation and amplification of erythroid cells. These data provide direct evidence for the model proposed by Iscove on the basis of responsiveness of hemopoietic cells to erythroid burst-promoting activity and erythropoietin.[24] Moreover, it seems to be interesting to examine whether blast cells can differentiate, when they are replated from the dishes containing IL-3 to dishes containing erythropoietin alone. In order to exclude the effect of IL-3 which might have been transferred to the replated culture, enough amounts of neutralizing antibodies to IL-3 would be necessary.

Single cell transfer experiments demonstrated that IL-3 plays a direct role in the differentiation of hemopoietic progenitors without the requirements for interactions with other cells. Without hemopoietic factors directed toward late stages of hemopoietic differentiation, the terminal differentiation of neutrophils, macrophages, eosinophils, and megakaryocytes was supported by IL-3. In summary, IL-3 supports the differentiation of multilineage hemopoietic progenitors, and the terminal differentiation of most hemopoietic lineages with the exception of the erythroid lineage.

ACKNOWLEDGMENT

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Monoclonal Antibody-Defined Functional Epitopes on the Adhesion-Promoting Glycoprotein Complex (CDw18) of Human Neutrophils

By W.J. Wallis, D.D. Hickstein, B.R. Schwartz, C.H. June, H.D. Ochs, P.G. Beatty, S.J. Klebanoff, and J.M. Harlan

We have evaluated the functional and immunochemical activities of three monoclonal antibodies (MoAbs) minimally reactive with adherence-defective neutrophils (PMN) from a patient with recurrent bacterial infections. In studies with normal PMN, MoAbs OKM1 and 60.1 both precipitate the same 165kd α-subunit (α_M) within an α-β heterodimer complex (CD11). The CD11 complex is part of a larger complex composed of four glycoproteins (CDw18) precipitated by MoAb 60.3, with properties suggesting that the CDw18 complex is equivalent to the Mac-1, LFA-1, p150, 95 glycoprotein family implicated in adherence-dependent leukocyte functions. PMN adherence to endothelium, spreading on surfaces, aggregation, and phagocytosis of zymosan particles were all inhibited in a dose-dependent fashion by MoAb 60.1 (analogous to previous studies with MoAb 60.3) while MoAb OKM1 had no effect. These findings unify previously disparate observations and suggest that a functionally active site on the adherence promoting glycoprotein complexes CD11 and CDw18 is distant from the α_M epitope recognized by MoAb OKM1 but closely associated with the α_M epitope recognized by MoAb 60.1 and the β-epitope (or epitope created by alpha-beta quarternary structure) recognized by MoAb 60.3.

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CONSIDERABLE attention has been directed toward a family of structurally related glycoproteins important in adherence-dependent leukocyte functions (reviewed in reference 1). Investigations with monoclonal antibodies (MoAbs) and neutrophils (PMN) have identified three such heterodimers with distinct α-subunits and a common β-subunit. Monoclonal antibodies recognizing antigens originally considered lymphocyte function-associated (also found on PMN) have been designated the LFA-1 series and bind a glycoprotein of M_r 177 kd designated α_L.[2-4] Monoclonal antibody OKM1 recognizes a glycoprotein of M_r 165 kd designated α_M on monocytes, PMN, and natural killer (NK) cells.[5,6] The α_M moiety is also recognized by MoAbs anti-Mo1,[6-8] OKM9,[4,9] and OKM10,[4,9] and appears to be identical to the receptor for complement product C3bi (CR3).[9,10] Reagents have recently been developed that identify the α-subunit (α_X) of a third heterodimer in this family, the p 150, 95 complex (See Note Added in Proof).[4] Additional MoAbs that may bind the common beta-subunit shared by all three heterodimers include MoAbs TS1/18,[2,4] 1B4,[9] and MHM23.[11,12]

Investigation has also been directed toward patients with recurrent bacterial infections whose PMN fail to express the adhesion-promoting molecules and fail to bind (or bind weakly) this family of MoAbs. Neutrophils from these patients are defective in adherence-dependent functions such as spreading, aggregation, chemotaxis, and phagocytosis.[13-19] We have reported two such patients whose PMN are adherence-defective and fail to react with reagents developed within our laboratories, MoAbs 60.1 and 60.3.[17,20] These antibodies are of considerable interest since binding of either to normal PMN induces defects in adherence-dependent functions similar to those observed in patients.[11,17,20,21] Studies with normal PMN have indicated that MoAb 60.1 recognizes a heterodimer with α-subunit similar to α_M (designated the CD11 complex by the Second International Workshop on Human Leukocyte Differentiation Antigens) and MoAb 60.3 precipitates at least three distinct polypeptides (designated the CDw18 complex).[11,22] The relationship between the glycoproteins recognized by MoAbs 60.1 and 60.3 and those identified by the LFA-1 and OKM1 reagents has previously been unclear since varying techniques of immunoprecipitation and gel electrophoresis in different laboratories have produced substantial variability in M_r.

The inhibitory effects of MoAbs 60.1 and 60.3 suggested to us that these reagents bind epitopes within the CD11 and CDw18 complexes centrally important in the adherence process. In this report we describe the results of studies comparing the functional and immunochemical activities of MoAbs 60.1 and OKM1. Our findings indicate that MoAbs 60.1 and OKM1 both immunoprecipitate the α_M subunit, but only MoAb 60.1 inhibits adherence-dependent PMN functions. These observations lend support to a recently proposed model of glycoprotein interaction associated with adherence-dependent leukocyte functions.[1]

MATERIALS AND METHODS

Neutrophils. Whole blood from normal healthy donors or the glycoprotein-deficient patient[17] was obtained by venipuncture and collected in heparin- (5 U/mL; Elkins-Sinn, Inc, Cherry Hill, NJ) coated syringes. Neutrophils were isolated by centrifuging whole blood on Ficoll-Hypaque (Pharmacia Fine Chemical, Piscataway, NJ) cushions, sedimentation of the Ficoll-Hypaque pellets in 3% dextran at room temperature, and lysis of residual red blood cells by hypotonic saline at 4 °C.[23]

Monoclonal antibodies. The preparation of murine monoclonal antibody (MoAb) 60.1 (IgG$_1$ kappa) and MoAb 60.3 (IgG$_{2a}$ kappa) has been described.[11,20] Previous studies indicate that MoAb 60.3 reacts with all human leukocytes and MoAb 60.1 reacts with all PMN, monocytes, some NK cells, and some lymphocytes. Monoclonal antibody OKM1 (murine IgG$_{2b}$; Ortho Diagnostics, Raritan, NJ) reacts with human PMN, monocytes, and NK cells. As previously reported,[20] murine MoAb H11 (IgG$_{2a}$-kappa) reacts with

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a 120 kd protein present on all PMN and some T lymphocytes and was used in these studies as a positive control. Murine MoAb 9E8 recognizes a phage membrane glycoprotein and has no specific reactivity with leukocytes or endothelium.[24] Monoclonal antibodies were purified by solid phase absorption on staphylococcal protein A columns (Pharmacia Fine Chemicals, Uppsala, Sweden)[25] and the concentration of purified antibody determined by Bradford assay.[26] In adherence and aggregation assays, both MoAbs 60.1 and OKM1 were prepared in PBS with final 0.02% sodium azide (J.T. Baker Co, Phillipsburg, NJ) and 0.1% bovine serum albumin (BSA; Irvine Scientific Co, Santa Ana, Calif). Earlier studies indicated that this concentration of azide had no effect on normal neutrophil adherence or aggregation. All other studies were conducted with MoAbs in PBS without azide or albumin.

Immunofluorescence flow cytometry. Immunofluorescence flow cytometry was performed using PMN from a normal donor and adherence-defective PMN from a patient (patient 1 in reference 13). Neutrophils were stained within siliconized polypropylene tubes (12 × 75 mm, Falcon, Oxnard, Calif). One million cells were suspended in 1 mL of phosphate buffered saline without calcium or magnesium (PBS−; Grand Island Biologic Co, Grand Island, NY) and washed with Waymouth's 752/1 media (Grand Island Biologic Co) and 5% pooled human plasma. Cells were incubated with saturating concentration of MoAb (40 ug/mL) for 30 minutes on ice, then fixed in freshly made 0.5% paraformaldehyde (Fisher Scientific Co, Pittsburgh, Pa) for 5 minutes, washed twice with 5% heat-inactivated fetal calf serum (FCS; Grand Island Biologic Co), and suspended in 25 uL of a saturating concentration of detecting antibody (fluorescein isothiocyanate (FITC)-rabbit antimouse immunoglobulin; TAGO, Burlingame, Calif) for 30 minutes on ice. Cells were washed once with 5% FCS and again with 1% BSA in PBS−. The final washed cell pellets were suspended in 0.5% paraformaldehyde and stored for up to three days in the dark at 4 °C prior to analysis. Analysis was performed by flow cytometry using a fluorescence-activated cell sorter (FACS 440, Becton Dickinson and Co, Sunnyvale, Calif) with a 50 micron orifice.

Immunoprecipitation and gel electrophoresis. Radioimmunoprecipitation studies were conducted using a previously published technique.[27] Briefly, neutrophils were surface labeled with ^{125}I by the lactoperoxidase method, then disrupted in cell lysis buffer [0.5% nonidet P-40, 0.15 mol/L NaCl, 0.05 mol/L EDTA, 0.05 mol/L Tris-HCl, pH 8.0, and 2 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co, St. Louis, Mo)]. Lysates were cleared of nuclei by centrifugation at 1500 × g for 10 minutes. Free iodide was separated from labeled proteins using Sephadex G-25 (Pharmacia). The amount of trichloroacetic acid (TCA) precipitable ^{125}I was determined for each lysate, and the concentrations of protein radiolabel equalized. Lysates were cleared of nonspecific binding proteins by incubation for 60 minutes with protein A sepharose (Pharmacia) which was then removed by centrifugation. Immunoprecipitation reactions were initiated by addition of 50 ug of MoAb coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). After incubation at 4 °C for 60 minutes, immune complexes were collected by centrifugation. Radiolabeled proteins were released by addition of sample electrophoresis buffer (62 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 0.02% bromophenol blue with 5% dithiothreitol) and heating to 100 °C for 5 minutes. Immunoprecipitated proteins were separated by electropheresis in 7% polyacrylamide gels in the presence of SDS under reducing conditions. Gels were dried and radiolabeled bands identified by radioautography. In experiments involving a second precipitating MoAb, cell lysates were extensively precleared by the first MoAb until no further radioactivity could be precipitated. The Sepharose-linked precipitating MoAb was then added to the precleared lysate with subsequent analysis as described above.

Spreading of adherent neutrophils on plastic. Normal neutrophils (2 × 10^6 cells/mL, 20 uL cell suspension) in Waymouth's medium in the presence of MoAb or BSA were incubated on plastic coverslips for 45 minutes at 37 °C. Coverslips were pretreated with 100 uL of phorbol myristate acetate (PMA; Sigma Chemical Company) (100 ng/mL in distilled water) and air dried. Coverslips and adherent cells were washed once. Coverslips were fixed with 1% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer pH 7.2, attached to teflon sheets, dehydrated through increasing concentrations of ethyl alcohol, critical point dried using liquid carbon dioxide, then attached to scanning electron microscope stubs using citron paste, coated with a layer of conductive heavy metal, and viewed in a JEOL 35C scanning electron microscope (Tokyo, Japan).

Neutrophil adherence to endothelial monolayers. Human umbilical vein endothelial cells (HUVE) were prepared by collagenase treatment of vessels as previously described.[28] Cells were maintained in RPMI 1640 (Grand Island Biologic Company, Grand Island, NY) with 20% FCS (Hyclone Laboratories, Logan, Utah) and were used in first or second passage only. Individual experiments were performed with HUVE of the same strain and passage number. Endothelial cells were plated in 11 mm wells in 48 well plates (Costar Cluster 3524, Cambridge, Mass) at 5 × 10^4 cells per well and grown to confluence over the course of two or three days. In studies contrasting MoAbs the assays were conducted concurrently with HUVE monolayers prepared from the same strain, passage, and flask. Prior to use in adherence assays, the plates were washed free of media and rinsed twice with Waymouth's medium without serum.

Neutrophils were radiolabeled with ^{51}Cr according to the method of Gallin et al.[29] Purified PMN in Hanks' balanced salt solution without Ca^{++} or Mg^{++} (Grand Island Biologic Company; HBSS−) (2 × 10^7 cells/mL) were incubated with 1 uCi ^{51}Cr per 10^6 cells (sodium chromate, 200 to 500 Ci/g, New England Nuclear, Boston, Mass) at 37 °C for one hour with periodic gentle agitation. Following incubation, the unbound ^{51}Cr was removed in three washes with HBSS. Labeled leukocytes were suspended in Waymouth's medium at a final concentration of 2 × 10^6/mL.

Neutrophil adherence to endothelium was measured by adding 0.25 mL of the purified ^{51}Cr-labeled PMN suspension (5 × 10^5 cells) to each well of the 48 well plate. Neutrophils were preincubated with appropriate concentrations of MoAbs for 30 minutes at 37 °C and added to the endothelial monolayer without prior removal of the unbound antibody. After the addition of leukocytes, control medium, PMA, or calcium ionophore A23187 (Calbiochem-Behring Corp, La Jolla, Calif) was added at the indicated concentration. The culture plates were gently agitated for 1 minute to promote mixing of the PMN suspension with the stimulant and contact with the endothelial monolayers. Plates were incubated for 45 minutes at 37 °C in a 5% CO$_2$ incubator. Medium and unattached cells were aspirated and the plate washed with Waymouth's medium. Aspirated cells and medium were collected in individual counting tubes and the monolayers and attached leukocytes were then lysed overnight with 1N NH$_4$OH. The lysate was aspirated, the plates washed once with lysing solution, and the combined lysate and wash fluid counted in a Beckman gamma spectrometer (Searle Analytic Inc, Division of G.D. Searle and Co, Des Plaines, Ill). Adherence was expressed as percentage of total ^{51}Cr counts remaining attached to the monolayer following incubation:

$$\%\text{ adherence} = \frac{^{51}\text{Cr cpm in lysate}}{\text{Total }^{51}\text{Cr cpm added}} \times 100$$

All samples were run in triplicate or quadruplicate.

Aggregometry. Aggregometry was performed by the nephelometric technique of Craddock et al[30] using a Chrono-log Model 330 platelet aggregometer with Model 702 recorder (Chrono-log Corp, Havertown, Pa). Neutrophils were suspended in HBSS− at a concentration of 2×10^7/mL. Three hundred fifty to four hundred uL aliquots were transferred to silicon-coated cuvettes containing teflon stirring bars. For experiments with MoAbs, PMNs were preincubated with 25 uL of MoAb or BSA for 10 minutes at 37 °C and further incubated for 5 minutes with 25 uL of cytochalasin B (Sigma Chemical Co; 100 ug/mL). The cuvettes containing the preincubated neutrophil suspension were then placed in the aggregometer and stirred at 900 rpm at 37 °C for one minute following the addition of 25 uL of a solution containing 14 mmol/L $CaCl_2$ and 7 mmol/L $MgCl_2$. Fifty uL of HBSS− or PMA in HBSS− (final concentration 100 ng/mL) were added and the resulting changes in light transmission recorded. A one-to-one mixture of neutrophils in HBSS− was used as the cell-poor calibration standard. Quantitation was achieved by measuring the area under the curve using a Hewlett Packard Model 9830A calculator and Model 9864A digitizer. Aliquots of PMN suspension were also removed at various time points for examination by phase contrast microscopy.

Neutrophil phagocytosis. Iodination by PMN was employed as an indirect measure of phagocytosis.[20] Into 12 × 75 mm polyethylene tubes was added 4×10^{-3} mol/L sodium phosphate buffer pH 7.4, 0.128 mol/L NaCl, 1.2×10^{-2} mol/L KCl, 10^{-3} mol/L $CaCl_2$, 2×10^{-3} mol/L $MgCl_2$, 8×10^{-6} mol/L NaI (4 mmol; 0.05 uCi ^{125}I), 2×10^{-3} mol/L glucose, 2.5×10^6 PMN, 0.5 mg opsonized zymosan, 0.25 mg BSA, and the MoAbs at the concentrations indicated, in a final volume of 0.5 mL. The zymosan (ICN Pharmaceuticals, Cleveland, Ohio) was suspended in water at a concentration of 10 mg/mL by homogenization at 4 °C, opsonized by incubation with an equal volume of pooled human serum for 20 minutes at 37 °C, washed, suspended, and then diluted ten-fold in the reaction mixture. The tubes were incubated for 20 minutes at 37 °C in a shaking water bath. The reaction was stopped by the addition of 1 mL of cold 10% TCA, the precipitate collected by centrifugation at 2,500 G for 5 minutes, and washed four times with 2 mL of 10% TCA. The tube containing the precipitate was assayed for radioactivity by gamma spectrometer (Packard). A blank containing the standard salt solution, iodide, and albumin was run with each experiment and the results subtracted from each experimental value. Less than 0.5% of the total added radioactivity was TCA-precipitable in the blank. A standard containing the total amount of ^{125}I in the reaction mixture was counted and the percent iodination was determined as follows:

$$\frac{\text{cpm experimental} - \text{cpm blank}}{\text{cpm standard}} \times 100$$

Results are expressed as the mean nmol of iodide converted to a TCA-precipitable form per 10^7PMN per hour. The results in each experiment were the average of duplicate determinations.

RESULTS

Immunofluorescence flow cytometry. Studies with neutrophils from a normal donor revealed low magnitude nonspecific binding of the negative control MoAb 9E8 and highly significant binding by MoAbs 60.1 and OKM1, which was comparable to that observed with the positive control MoAb H11 (Fig 1A). In contrast, neutrophils from a patient with recurrent infections and adherence-defective phagocytes demonstrated greatly decreased binding of both MoAbs OKM1 and MoAb 60.1 (Fig 1B). Binding of positive H11 and negative (9E8) control MoAbs to the adherence-defective PMN was identical to that observed with normal PMN.

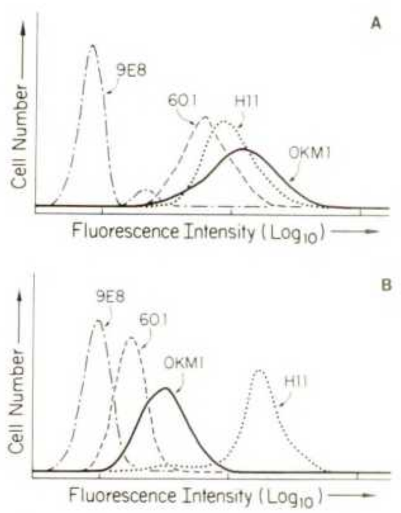

Fig 1. **Monoclonal antibody binding to normal neutrophils (A) and a patient's adherence-defective neutrophils (B). MoAb OKM1 binds a PMN heterodimer of M, 165 kd and 95 kd. MoAb 60.1 binds a PMN heterodimer of M, 165 kd and 95 kd. MoAb H11 reacts with a 120 kd protein present on all PMN and some T lymphocytes. MoAb 9E8 reacts with a phage glycoprotein and has no reactivity with leukocytes. The binding of MoAb OKM1, MoAb 60.1, and MoAb H11 positive control are compared with MoAb 9E8. Binding of MoAbs OKM1, 60.1, and H11 are significant in normal neutrophils. Patient neutrophils fail to bind significantly both OKM1 and 60.1.**

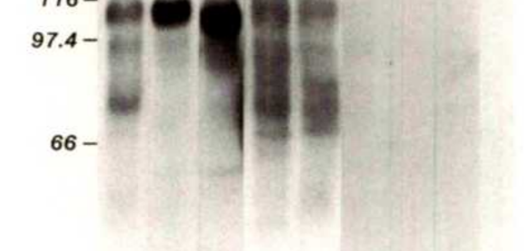

Fig 2. **Immunoprecipitations from ^{125}I-labeled human PMN. Neutrophil lysates were incubated only with precipitating MoAb (lanes 1, 2, and 3) or incubated first with the designated preclearing MoAb, then exposed to the precipitating MoAb. All immunoprecipitates were subjected to 7% SDS-PAGE and autoradiography. Molecular weight markers shown on the left are listed as kd. Preclearance studies with control MoAb 60.5, which is directed to a Class I HLA-framework antigen present on all peripheral blood leukocytes,[11] yielded results identical to those obtained when PMN lysates were preincubated with buffer alone (data not shown).**

Immunoprecipitation and gel electrophoresis. Immune precipitation and SDS-PAGE of ^{125}I surface-labeled neutrophils demonstrated that MoAbs OKM1 and 60.1 precipitated identical heterodimers consisting of an α-subunit of approximately 165 kd (α_M) and a β-subunit of approximately 110 kd (Fig 2). MoAb 60.3 precipitated the same α-chain (α_M) and β-chain as observed with 60.1 and OKM1, along with two additional molecules with approximate molecular weights of 177 kd (α_L) and 150 kd (α_X).

Preclearance of the PMN lysates with MoAb 60.1 or OKM1 removed only the 165 kd molecule from the complex precipitated by MoAb 60.3 (lanes 4 and 5). Preclearance with MoAb 60.1 removed the OKM1 heterodimer and preclearance with OKM1 removed the 60.1 heterodimer (lanes 6 and 7). Preclearance with MoAb 60.3 removed all of the subunits precipitated by 60.1 and OKM1 (lanes 8 and 9). Preclearance studies with control MoAb 60.5, which is directed to a Class I HLA-framework antigen present on all peripheral blood leukocytes,[11] yielded results identical to those obtained when PMN lysates were preincubated with buffer alone (data not shown).

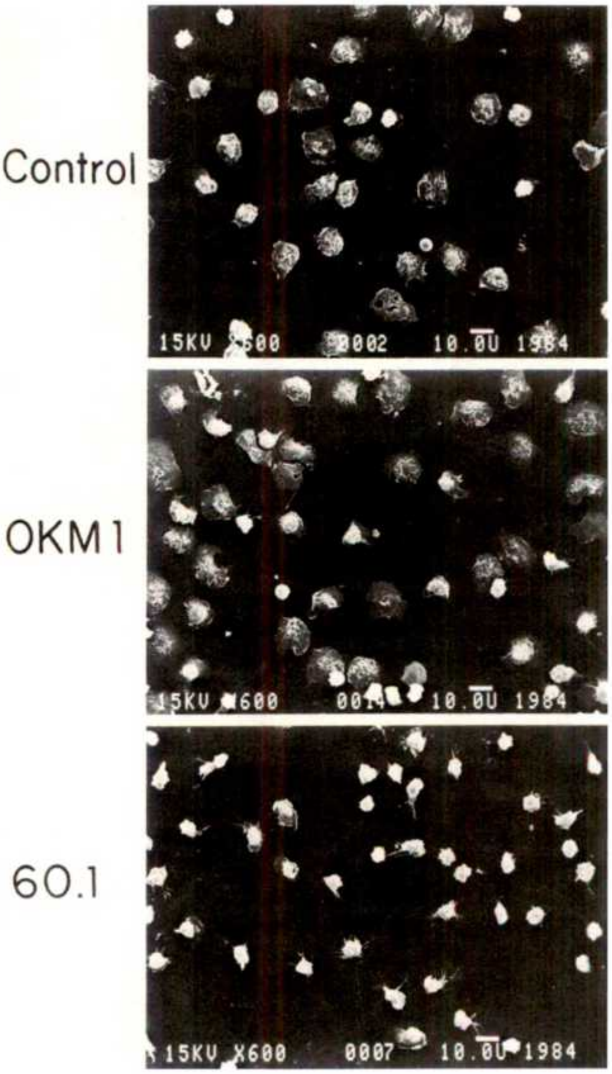

Fig 3. Scanning electron micrographs of PMA-activated neutrophils adherent and spread on polystyrene coverslips. Following a 15-minute incubation with MoAb (40 ug/mL) or BSA (40 ug/mL), neutrophils were incubated on PMA-coated plastic for 45 minutes at 37 °C. Only MoAb 60.1 inhibited PMN spreading.

Effect of MoAbs on spreading of stimulated neutrophils. Neutrophils from normal donors treated with phorbol ester adhered and spread on polystyrene (tissue culture plastic) coverslips. This process was not affected by saturating concentrations of MoAb OKM1 (Fig 3). In marked contrast, normal PMN treated with MoAb 60.1 adhered minimally and failed to spread. PMN assayed in the presence of MoAb 60.1 simulated cells from patients with adherence-defective PMN.[17]

Neutrophil adherence to endothelium. Basal (unstimulated) adherence of ^{51}Cr-labeled PMN in serum-free medium on HUVE monolayers was of low magnitude (11% ± 4%). The addition of PMA to the adherence assay (final concentration 100 ng/mL) markedly augmented neutrophil adherence (88% ± 7%), as did the addition of calcium ionophore A23187 (44% ± 6%). Neutrophil adherence stimulated by PMA or A23187 was not significantly inhibited by coincubation with MoAb OKM1 or BSA. In marked contrast, the augmentation of neutrophil adherence by either PMA or A23187 was significantly inhibited in a dose-dependent fashion by MoAb 60.1 (Fig 4).

Neutrophil aggregation. Neutrophils from normal donors aggregated in response to phorbol ester. Addition of BSA or MoAb OKM1 did not significantly modify PMA-induced aggregation. In contrast, PMA induced minimal aggregation of neutrophils treated with saturating concentrations of MoAb 60.1. Neutrophils incubated with MoAb 60.1 simulated the responses of adherence-defective leukocytes (Fig 5).

Phagocytosis. Figure 6 demonstrates the effect of MoAb 60.1 and OKM1 on iodination by normal PMN. No significant inhibition of iodination was produced by 40 ug/mL of OKM1 under conditions in which MoAb 60.1 was markedly inhibitory, with half-maximum inhibition occurring at a concentration of < 1 ug/mL. The close correlation between the effects of MoAbs 60.1 and 60.3 on iodination and on phagocytosis when the particulate stimulus zymosan was employed[20] and the absence of an effect of MoAb 60.3 on iodination when the soluble stimulus PMA was used[17] suggests that iodination is a measure of the phagocytosis of zymosan under the conditions used here.

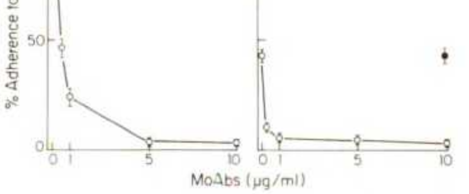

Fig 4. Dose-dependent inhibition of stimulated PMN adherence to cultured vascular endothelium by MoAb 60.1 (open circle). MoAb OKM1 (solid circle) had no apparent effect on stimulated PMN adherence. (A) PMA (100 ng/mL); (B) A23187 (10 umol/L).

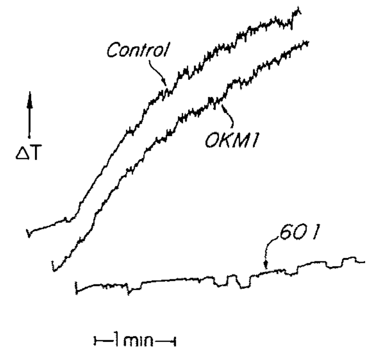

Fig 5. Inhibition of PMA-induced PMN aggregation by MoAb 60.1 (40 ug/mL) and the lack of inhibition by MoAb OKM1 (40 ug/mL).

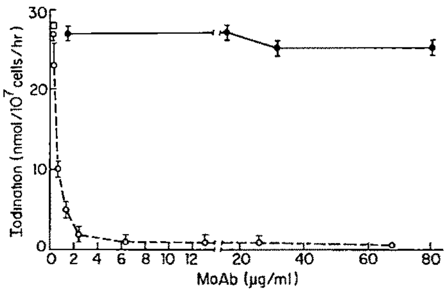

Fig 6. MoAb 60.1 induces a dose-dependent inhibition of PMN phagocytosis of opsonized zymosan as assayed by iodination. MoAb OKM1 is without significant effect.

DISCUSSION

In this report we describe the results of studies comparing the functional and immunochemical activities of MoAbs 60.1 and OKM1. Both reagents demonstrated little reactivity with adherence-defective PMN from a patient with recurrent bacterial infections. This observation confirms previous studies[19,20] and indicates that the epitopes bound by these reagents were either absent or expressed at insignificant levels in patient cells. Immunochemical studies with normal PMN established that both MoAbs bind the same alpha subunit glycoprotein (α_M) within the α-β heterodimer complex (CD11) and that this molecule is part of a larger complex composed of four glycoproteins (CDw18) immunoprecipitated by MoAb 60.3. Assays of adherence-dependent PMN functions were conducted in the presence of MoAbs OKM1 and 60.1. PMN adherence to endothelium, spreading, aggregation, and phagocytosis were all inhibited in a dose-dependent fashion by MoAb 60.1, while MoAb OKM1 had no effect. Thus, only MoAb 60.1 binds a site on the α_M molecule which is critical to the adhesion process.

Glycoprotein deficiency in adherence-defective PMN has previously been defined by minimal reactivity with a variety of murine MoAbs (reviewed in reference 1) and by near absence of selected glycoproteins on electrophoretic analysis of PMN lysates. Missing glycoproteins have been variably reported as 110kd,[15] 180kd,[18,31,32] 150kd,[14,17,21] and 138kd.[19] Many investigators feel that different patients with similar clinical manifestations probably share the same glycoprotein deficiency, and variations in reported molecular weights probably reflect different gel conditions in different laboratories.[1] Recent evidence suggests that adherence-defective PMN fail to synthesize the β-subunit that is necessary for leukocyte surface expression of the α_L, α_M, and α_X subunits.[33] It has been proposed that inheritance of this biochemical and clinical defect is either autosomal recessive[33] or X-linked recessive.[15]

Monoclonal antibodies indirectly map the functionally active site in the adhesion-promoting glycoprotein complex (reviewed in reference 1). We have previously reported that MoAb 60.3 inhibits PMN adherence to endothelium, PMN spreading, phagocytosis of C3bi-opsonized particles, aggregation, and chemotaxis.[11,17,20,21] In this report we have demonstrated that MoAb 60.3 precipitates four molecules with M_r compatible with the α_L, α_M, α_X, and β-chains. This observation suggests that the CDw18 complex is equivalent to the Mac-1, LFA-1, p150, 95 glycoprotein family. Monoclonal antibody 60.3 removed the α_M molecules recognized by 60.1 or OKM1, but MoAbs 60.1 and OKM1 removed only the α_M molecule from the complex recognized by 60.3. Monoclonal antibody 60.3, like MoAbs TS1/18,[4] MHM23,[12] and 1B4,[9] may bind a site on the common β-subunit.[22] Indirect support for this interpretation is found in the observation that MoAb 60.3 binds leukocytes of both myeloid and lymphoid lineages (all expressing β-subunits). Although we cannot rule out the possibility of a cross-reacting epitope on all four subunits, we feel it more likely that the epitope recognized by MoAb 60.3 resides on the common β-subunit. Alternatively, the epitope recognized by MoAb 60.3 may result from the quarternary structure arising from noncovalent association of α- and β-subunits. Supporting this latter interpretation, MoAb 60.3 (like MoAb 1B4[9]) fails to bind to its precipitated polypeptides in their dissociated state (data not shown). Other workers have shown that MoAb anti-Mo1 precipitates the α_M glycoprotein and blocks adherence, spreading, aggregation, and phagocytosis.[34,35] Inhibition of adherence-dependent functions by MoAbs 60.1, 60.3, and anti-Mo1 (but not OKM1) implies that a functionally active site is distant from the epitope recognized by MoAb OKM1 but closely associated with the α_M epitopes recognized by MoAbs 60.1 and anti-Mo1 and the β-epitope (or β-α quarternary epitope) recognized by MoAb 60.3.

The function of adhesion-promoting glycoproteins and the mechanism by which MoAbs inhibit adherence-dependent functions is currently unclear. Previous studies have shown that PMN stimulation with agents such as phorbol ester or calcium ionophore augments PMN adherence to surfaces and simultaneously increases surface expression of epitopes reactive with MoAbs, Mo1, LFA-1, and 60.3.[21,34,36] This results in part from surface recruitment of additional glycoproteins from cytoplasmic granules.[36] PMN-stimulating agents may perhaps further augment adherence by inducing a qualitative change in surface glycoproteins (eg, phosphorylation or proteolytic cleavage) that renders these molecules more intrinsically adhesive, or promotes interaction of glycoproteins with cellular structures (eg, cytoskeleton) involved in adherence phenomena. We presume that MoAb inhibition of adherence-dependent PMN functions results from direct binding or steric hindrance at a functionally important site on the α_M molecule. The results we have presented, however, do not rule out the possibility that MoAb 60.1 inhibition is caused by capping of α_M molecules or by linkage of the α_M moiety with Fc receptors. Inhibition of PMN adherence is unlikely to reflect nonspecific interaction of MoAb with the cell surface membrane since other MoAbs reactive with the PMN surface do not inhibit adherence-dependent functions.[1,11,20,21]

Previous studies with C3bi-coated erythrocytes have demonstrated that adherence-defective PMN from patients with recurrent infections also lack C3bi receptors (CR3).[16,19,20] The 165kd membrane glycoprotein designated α_M appears to be closely associated with or identical to CR3. Antibody blocking studies with normal PMN or monocytes have shown that CR3 function is retained in the presence of OKM1, OKM9, 1B4, and TS1/18,[9] and specifically blocked by MoAbs 60.1, 60.3, anti-Mo1, anti-Mac-1, and OKM10.[9,10,20] Since MoAbs 60.1, 60.3, and anti-Mo1 all block adherence-dependent PMN functions (and OKM1 does not), these data suggest that epitopes mediating CR3 function and epitopes mediating adherence-dependent functions are either in close spatial linkage or identical.

NOTE ADDED IN PROOF

Reagents that identify the α-subunit (α_X) of the p 150, 95 heterodimer have recently been described in the following: Lanier LL, Arnaout MA, Schwarting R, Warner NL, Ross GD: p 190/95, third member of the LFA-1/CR$_3$ polypeptide family identified by anti-Leu M5 monoclonal antibody. Eur J Immunol 15:713, 1985, and Springer TA, Miller LJ, Anderson DC: p 150, 95, the third member of the Mac-1, LFA-1 human leukocyte adhesion glycoprotein family. J Immunol 136:240, 1986

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Development of a Radioimmunoassay for the Fibrinogen-Derived Peptide Bβ1-42

By Jeffrey I. Weitz, James A. Koehn, Robert E. Canfield, Sharon L. Landman, and Robert Friedman

The peptide Bβ1-42 is the initial cleavage product of plasmin-mediated proteolysis of the NH$_2$-terminal region of fibrinogen or fibrin I, while β15-42 is the major fragment released by plasmin degradation of fibrin II. Numerous studies have described the measurement of plasma Bβ1-42 levels as an index of plasmin activity. Previous assays were indirect and included quantitation of thrombin-increasable fibrinopeptide B immunoreactivity (TIFPB) or measurement of β15-42 with an antiserum (132) which cross-reacted with Bβ1-42. We report on a new antiserum (R142) directed against Bβ1-42 which does not cross-react with β15-42 or fibrinopeptide B. Employing this antiserum, a specific assay for Bβ1-42 was developed. This assay was used to measure plasmin-mediated Bβ1-42 release from fibrinogen and its subsequent proteolysis by thrombin. The selectivity constant (Kcat/Km) for thrombin cleavage of the Bβ14-15 bond of Bβ1-42 was 10^5-fold less than that for proteolysis of the same bond in the intact fibrinogen molecule, thus explaining the stability of this peptide in the presence of thrombin activity in the blood. Similarly, the selectivity constant for plasmin cleavage of the Bβ21-22 bond of Bβ1-42 was 140-fold less than that for proteolysis of the Bβ42-43 bond of fibrinogen, indicating that secondary plasmin attack of the Bβ1-42 molecule is not physiologically important. The specific Bβ1-42 assay provided excellent recovery of peptide added to blood or plasma. Comparison of Bβ1-42 levels with TIFPB values in 37 patient samples yielded good correlation over a wide range of levels ($r^2 = 0.91$). The median plasma Bβ1-42 level in 15 normal individuals was 1.2 pmol/mL. This is similar to the previously reported normal range for TIFPB (1 to 4 pmol/mL) but is higher than the normal level of 0.4 pmol/mL reported with the assay employing antiserum 132. This discrepancy reflects rapid removal of Arg (Bβ42) by plasma carboxypeptidase activity resulting in 50% loss of Bβ1-42 immunoreactivity with antiserum 132 but no loss with R142. To circumvent this problem, we have developed a β15-42 antiserum (R154) which, like antiserum 132, cross-reacts with Bβ1-42. However, Bβ1-42/β15-42 immunoreactivity with R154 is stable in the presence of carboxypeptidase activity. Thus, these studies confirm the utility of measurement of plasma Bβ1-42 as an index of plasmin activity. Further, in vitro studies of Bβ1-42 generation by recombinant tissue-plasminogen activator suggest that radioimmunoassays for the plasmin-derived fibrinopeptides may provide a method for investigating the fibrin(ogen)olytic activity of thrombolytic agents.

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THE balance between thrombin and plasmin action on fibrinogen has been postulated to be an important determinant of thrombosis.[1] Specific and quantitative analysis of this balance can be assessed by measurement of the products of this proteolysis. Thrombin acts on fibrinogen in a two-step fashion.[2] The first step releases fibrinopeptide A (Aα1–16) from the NH$_2$-terminal region of the Aα-chain producing an intermediate known as fibrin I, while the second step involves cleavage at the NH$_2$-terminal region of the Bβ-chain, releasing fibrinopeptide B (Bβ1–14) and producing fibrin II. In the initial digestion of fibrinogen by plasmin, large segments of the COOH-terminal Aα-chain and Bβ1-42, from the NH$_2$-terminal region of the Bβ-chain, are cleaved and fragment X is formed.[3-6] Fibrin II forms more compact fibers with greater tensile strength than fibrin I,[7] and because it forms α-polymer cross-links, is less susceptible to lysis by plasmin than fragment X.[8] It has been hypothesized, therefore, that the amount of fibrin II formed is an important determinant of thrombosis and that the balance between fibrin II and fragment X formation determines the pathophysiologic consequences of fibrin I generation.[1]

Unlike the Aα and γ-chains, the NH$_2$-terminal region of the Bβ-chain of fibrinogen contains cleavage sites for both thrombin (Bβ14-15 bond) and plasmin (Bβ42-43 bond). The measurement of the products released by these cleavages, fibrinopeptide B and Bβ1-42, respectively, reflects the balance between thrombin and plasmin action on fibrin I. Methods to detect circulating levels of fibrinopeptide B have been described,[9,10] but to date only indirect methods to measure Bβ1-42 have been available.[11,12]

In this study, several β-chain fragments from the NH$_2$-terminal region of human fibrinogen were purified. The peptides Bβ1-42 and β15-42 were selected as immunogens because they are most characteristic of the plasmin-derived fragments from this region. Each peptide was coupled to bovine serum albumin by carbonyldiimidazole, and polyclonal antibodies were produced in rabbits. These antisera were then characterized using radioimmunoassay techniques and their specificity explored using a panel of purified human β-chain fragments. Radioimmunoassays employing these antisera provide useful tools for in vitro and in vivo study of plasmin-mediated proteolysis of fibrinogen and fibrin.

MATERIALS AND METHODS

Preparation of Fibrinogen and Plasmin

Human fibrinogen (Grade L, Kabi, Sweden) was purified as previously described.[13] Plasminogen was prepared from fresh frozen plasma by affinity chromatography, further purified by ion-exchange chromatography, and activated with insolubilized urokinase as described previously.[13]

Purification of Immunogens

N-DSK was prepared by digesting human fibrinogen (Grade L, Kabi) with cyanogen bromide followed by gel filtration over a G-100

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(Pharmacia Fine Chemicals, Uppsala, Sweden) column (4.0 × 200 cm) as described previously.[14] The N-DSK was dissolved in Tris-buffered saline (0.05 mol/L Tris, 0.1 mol/L NaCl, pH 8.6) to a concentration of 20 mg/mL, and digested with 0 01 CU human plasmin/mg N-DSK for 2 hours at 37 °C. The reaction was stopped by the addition of acetic acid (final concentration 10%) and the digest was gel filtered over a Sephadex G-50 superfine (Pharmacia) column (2 5 × 150 cm) in 10% acetic acid. The region containing Bβ1-42 was identified using high performance liquid chromatography (HPLC) and its composition confirmed by amino acid analysis. The Bβ1-42 pool was lyophilized and further purified using semipreparative high performance liquid chromatography, as described elsewhere.[15]

Purification of β-chain Fragments

Bβ1-118 was prepared as previously described.[13] β22-42 was obtained by exhaustive plasmin proteolysis of purified Bβ1-42. The Bβ1-42 was dissolved in Tris-buffered saline, pH 7.4, and digested with human plasmin (1 CU plasmin/mg Bβ1-42) for 3 hours at 37 °C. The reaction was stopped by the addition of phosphoric acid (final pH <4) and the products were separated by HPLC.

β15-42 was prepared by digesting purified Bβ1-42 with human thrombin. Bβ1-42 in Tris-buffered saline, pH 7 4, was incubated with human thrombin (NIH Lot H-1, 60 U/mg Bβ1-42) for 60 minutes at 37 °C. Fibrinopeptide B (FPB) and β15-42 were then separated and further purified by HPLC as previously described.[16] β12-19 and β12-42 were prepared from chymotryptic digests of Bβ1-42. The Bβ1-42 dissolved in Tris-buffered saline, pH 7.4, was digested with chymotrypsin (Worthington Biochemical Corp, Freehold, NJ) using an enzyme to substrate ratio of 1:100 (by weight). After 5 minutes' digestion at 37 °C, phosphoric acid was added to half the reaction mixture (final pH <4) and the products were separated by HPLC. The remainder of the sample was digested for 60 minutes at 37 °C, phosphoric acid was added to stop the reaction, and HPLC was used to separate the products Amino acid analysis demonstrated that the major fragments produced by limited digestion were Bβ1-11 and β12-42, while those produced by exhaustive digestion were Bβ1-11, β12-19, and β20-42.

High Performance Liquid Chromatography (HPLC)

Analytical and semipreparative HPLC were performed using a Waters Associates liquid chromatograph equipped with a Model 660 solvent programmer, two Model 6000A solvent delivery systems, a Waters automatic injector Model 710A, and a Waters data module. Peptides were monitored using a Model 450 variable wave length absorbance detector set at 220 nm. The column employed for all analyses was a μ-Bondapak C_{18} (3 9 mm i.d. × 30 cm) from Waters Associates, which was maintained at 37 °C using a water bath and thermal jacket. Peptide samples were eluted from the μ-C_{18} column either isocratically or using gradients containing various concentrations of acetonitrile in the mobile phase. Buffers used in the chromatography included 0.1% phosphoric acid (HPLC grade, Fisher Scientific Co, Fairlawn, NJ), pH 2.1 (buffer A), and 0.1% phosphoric acid containing 50% acetonitrile, pH 2.1 (buffer B). Milligram quantities of Bβ1-42 and β15-42 were purified using a larger, semipreparative μ-Bondapack C_{18} column (7.8 mm i.d. × 30 cm) also from Waters Associates. The peptides were eluted with acetonitrile gradients produced by a buffer system prepared with 0.1% Trifluoroacetic acid (Pierce Chemical Co, Rockford, Ill), pH 2.1 (buffer A), and 0.1% Trifluoroacetic acid containing 50% acetonitrile, pH 2.1 (buffer B) and were detected at 230 nm. All buffers were prefiltered through a preparative μ-Bondapak C_{18} Porasil B column (7.8 mm i.d. × 61 cm) from Waters Associates and degassed prior to use.

Amino Acid Analysis

Fractions to be analyzed were hydrolyzed in 6N HCl in vacuo for 24 hours at 110 °C and analyzed using standard techniques on a Beckman Model 121 MB amino acid analyzer.[17]

Immunization

Purified Bβ1-42 and β15-42 were conjugated to 2 × crystallized bovine serum albumin (Sigma Chemical Co, St. Louis, Mo) using N-N'-carbonyldiimidazole (Pierce). In a 5 mL reaction vial, 3 mg of each peptide was suspended in 3 mL of anhydrous dimethylformamide (Pierce) containing 10 mg carbonyldiimidazole and the mixture was bubbled with nitrogen for 15 minutes. Five mg of BSA was added and the reaction was continued with stirring and nitrogen bubbling for 30 minutes at 23 °C. The reaction mixture was then desalted on a Sephadex G-25 (Pharmacia) column (2.5 × 40 cm) in 10% acetic acid and lyophilized. The immunogens were resuspended in phosphate buffered saline (0.9% NaCl containing 0.01 mol/L phosphate, pH 7.4) to a concentration of 2 mg/mL and vortexed thoroughly with 3 parts Freund's Complete Adjuvant (Calbiochem-Behring Corp, La Jolla, Calif) until a thick emulsion formed.

White male New Zealand rabbits were immunized with 200 μg of the conjugated peptide using the multiple intradermal injection technique.[18] Ten weeks after the primary injection, each rabbit was boosted with 100 μg of the immunogen given intramuscularly into the haunches. Blood was collected at 8 weeks and every 2 weeks thereafter. To detect an immune response, Bβ1-42 and β15-42 were radioiodinated at residue Bβ41 (tyrosine) with ^{125}I (Amersham Corp, Arlington Heights, Ill) to a specific activity of 30–50 μCi/μg by the chloramine-T method,[19] and specific binding of these tracers was determined.

Radioimmunoassays

TIFPB, fibrinopeptide A (FPA), and fibrinopeptide B were assayed as previously described.[9,11,20] For assay of Bβ1-42 or β15-42, dilutions of standard, test samples, antiserum, and tracer were made in 0.01 mol/L phosphate buffered saline, pH 7.4, with 1% (v/v) normal rabbit serum, 0.1 mg/mL lysozyme (Worthington), and 350 KI units/mL aprotinin (Mobay Chemical Corp, New York, NY) Each assay tube contained 650 μL final volume consisting of the following, in order of addition· 500 μL of standard concentration of peptide to be measured or a dilution of test sample, 100 μL of antiserum (sufficient to bind 25% to 35% of the total bindable counts), and 50 μL of tracer (about 10,000 cpm). After 24 hours incubation at 4 °C, tracer bound to antibody was separated by a second antibody technique. Sheep antirabbit IgG, in quantities capable of binding all of the rabbit IgG, and diluted in 0.01 mol/L phosphate-buffered saline, pH 7 4, supplemented with 20 mg/mL polyethylene glycol 6,000 (Fisher Scientific Co, Springfield, NJ) was added. The tubes were then incubated for 30 minutes at 23 °C, diluted with 2 mL of 0.9% NaCl, and centrifuged at 4,000 g for 20 minutes at 4 °C. The supernatant fluid was aspirated and the pellet was counted for 1 minute on a Rackgamma counter (#1270, LKB Instruments, Rockville, Md).

Kinetic Studies

Because of the specificity of the Bβ1-42 antiserum (see below), kinetic data describing plasmin-mediated cleavage of the Bβ42-43 bond of fibrinogen and subsequent thrombin proteolysis of the Bβ14-15 bond of Bβ1-42 could then be estimated. Fibrinogen (0 1 to 12 μmol/L) was digested with plasmin (01 CU/mL) at 37 °C and the initial velocity of Bβ1-42 cleavage calculated using appearance of Bβ1-42 immunoreactivity during the first 5 minutes of the reaction Less than 10% of total substrate was digested during this time. Purified Bβ1-42 (1.0 to 100 μmol/L) was then digested with thrombin (1.0 U/mL) at 37 °C and initial velocity calculated by measuring disappearance of Bβ1-42 immunoreactivity during the first 5 minutes of the reaction. Again, less than 10% of the substrate had been consumed during this time.

Blood Collection and Processing

Blood from normal individuals was donated by laboratory personnel and house officers who were in apparent good health. Samples were collected from an antecubital vein using a 21-gauge butterfly needle. For Bβ1-42 and TIFPB measurements, 4.5 mL blood was collected into a plastic tube containing 0.5 ml of anticoagulant solution consisting of 1,000 U/ml Trasylol, 1,400 U/ml heparin, 0.01 mol/L adenosine, and 0.02 mol/L theophylline in HEPES-buffered saline, pH 7.4. For in vitro recovery experiments, blood was collected into a plastic syringe and transferred into a plastic tube containing 1/10 the volume of above anticoagulant solution, immediately transferred to a 50 mL conical plastic tube, kept on ice, and mixed gently. The anticoagulated blood was either transferred into 15 × 90 mm polystyrene tubes containing different concentrations of Bβ1-42, mixed gently, and then centrifuged at 1,700 g at 4 °C for 20 minutes, and the plasma pipetted off, or the blood was centrifuged as above and the plasma was then transferred into 15 × 90 mm polystyrene tubes containing different concentrations of Bβ1-42 and mixed gently. In each case, within 30 minutes of blood collection, 2 mL plasma was precipitated with an equal volume of denatured ethanol (Fisher) to remove the fibrinogen, evaporated to dryness, and reconstituted with distilled water as previously described.[11] For in vitro studies of Bβ1-42 generation by recombinant tissue-plasminogen activator (rt-PA), venous blood from normal volunteers was obtained in plastic syringes containing 3.8% trisodium citrate (9:1 v/v) Samples were centrifuged at 1,700 g for 20 minutes at 4 °C and the plasma pipetted off and immediately used for the experiments (see below).

Comparison of Bβ1-42 and TIFPB Assays

Thirty-seven stored ethanol supernatants taken from patients with various clinical disorders were thawed and assayed for Bβ1-42 (using antiserum R142) and for TIFPB. The coefficient of covariance was calculated as described by Sokal and Rohlf[21]

Studies of Stability of Bβ1-42 in Plasma

Incubation of Bβ1-42 in Plasma. Bβ1-42 (30 pmol/mL) was incubated with human plasma at 23 °C for one hour. Aliquots were removed at intervals and the plasma was precipitated with ethanol (50% final concentration), the ethanol evaporated, and the precipitate dissolved in distilled water as previously described.[11] The reconstituted samples were then assayed for Bβ1-42 immunoreactivity using antisera R142, R154, and 132 (a gift of Dr B. Kudryk, New York Blood Center).

Effect of Carboxypeptidase B on the Immunoreactivity of Bβ1-42 in Buffer Solution Bβ1-42 at a final concentration of 20 pmol/mL was incubated at 37 °C in 0.15 mol/L Tris-buffered saline (0.1 mol/L NaCl and .05 mol/L Tris, pH 7.4) with and without porcine pancreas carboxypeptidase B (Worthington). The enzyme activity was determined immediately before use by measuring hydrolysis of hippuryl-L-lysine (Sigma). Final carboxypeptidase concentrations of 1.25 × 10^{-3} and 1.25 × 10^{-4} units/pmol of Bβ1-42 were employed. The reaction was terminated by adding 10 μL of a stock solution of 2.0 mol/L O-phenanthroline (Sigma) in absolute ethanol (final concentration 0.01 mol/L) and Bβ1-42 immunoreactivity was measured with antisera R142, R154, and 132.

The effects of carboxypeptidase B treatment of Bβ1-42 were also assessed by HPLC. Bβ1-42 (50 μg) in Tris-buffered saline, pH 7.4, was incubated at 23 °C for 60 minutes with a final carboxypeptidase B concentration of 1.0 × 10^{-4} units/pmol of Bβ1-42. Under the same conditions the experiment was repeated with a carboxypeptidase B concentration of 1.0 × 10^{-3} units/pmol of Bβ1-42.

Generation of Bβ1-42 in Vitro

Recombinant human tissue plasminogen activator (rt-PA; Genentech, San Francisco, Calif) diluted in Tris-buffered saline, pH 7.5, with 0.1 mg/mL Tween 20 (Fisher), was incubated at 37 °C with 2 mL citrated plasma. At intervals, aliquots were removed, aprotinin (100 U/mL) was added, and the samples were precipitated with 50% ethanol, processed as described above and assayed for Bβ1-42 (using antiserum R142) and for FPA The experiment was repeated first using citrated plasma that had been preincubated with 0.1 U/mL of a purified fraction of reptilase (a gift of Dr B. Blomback) for 10 minutes at 23 °C before the addition of rt-PA, and then with citrated plasma to which reptilase and rt-PA were added simultaneously. The reptilase produced a visible clot within 90 seconds.

RESULTS

Characterization of the Antisera

Purified Bβ1-42 and β15-42 were conjugated to bovine serum albumin and used as immunogens in rabbits. Two of four rabbits receiving the Bβ1-42 immunogen responded. One antiserum, designated R142, showed the highest titer and was employed in this study. Fig 1A shows the reactivity of this antiserum with the various β-chain competitors. There was complete cross-reactivity between Bβ1-42 and Bβ1-21 while FPB, β15-42, and the other β-chain competitors (β12-42, β12-19, β22-42) did not react.

In contrast, although eight rabbits received the β15-42 immunogen, all produced low titer antisera after repeated booster immunizations and four died of unknown causes during a four-month period. Only one antiserum, designated R154, exhibited a sufficient titer to be employed in a radioimmunoassay. Competitive binding studies using this antiserum and ^{125}I-β15-42 as tracer are shown in Fig 1B. There was complete cross-reactivity among Bβ1-42, β15-42, and β22-42 while FPB and Bβ1-21 exhibited little reactivity.

Plasmin Cleavage of the β-chain of Fibrinogen

In a previous report[13] the sequence of plasmin cleavage of the NH_2-terminal region of the Bβ-chain of fibrinogen was studied using HPLC methods. Because of the specificity of

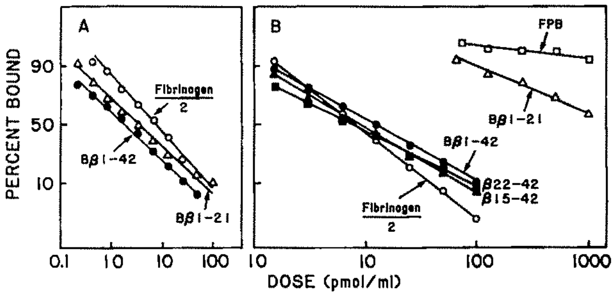

Fig 1. Competitive binding curves. (A) Using antiserum R142 and ^{125}I-Bβ1-42 as tracer. (B) Using antiserum R154 and ^{125}I-β15-42 as tracer.

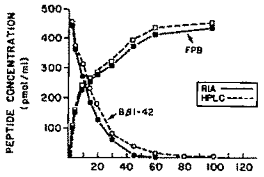

Fig 2. Thrombin proteolysis of Bβ1-42. Purified Bβ1-42 (22 μg/mL) was digested with thrombin (1.0 U/mL) and radioimmunoassays (RIA), employing antisera R142 and R22, were used to measure Bβ1-42 and FPB, respectively. HPLC was used in parallel to quantitate these peptides.

antiserum R142, these results could now be confirmed using radioimmunoassay, as well as HPLC techniques, to monitor Bβ1-42 release. Immunoreactivity, as measured using antiserum R142, reached a maximum between 40 and 60 minutes reflecting quantitative peptide release. HPLC was employed to separate and quantify the β-chain fragments. Bβ1-42 levels, as measured using HPLC, increased in parallel with immunoreactivity during the early stages of the digestion, reached a maximum, and then began to decline. As reported previously,[13] increasing levels of Bβ1-21 were seen following the initial Bβ1-42 release. The radioimmunoassay utilizing antiserum R142, which cross-reacts completely with Bβ1-42 and Bβ1-21, measured both of these peptides.

Kinetic Studies

Figure 2 illustrates thrombin proteolysis of purified Bβ1-42. The disappearance of Bβ1-42 immunoreactivity, measured using antiserum R142, was mirrored by the subsequent appearance of FPB immunoreactivity quantitated with R22 antiserum. HPLC measurements of these two peptides were in good agreement with the immunochemical data. The estimated kinetic constants for plasmin-mediated cleavage of the Bβ42-43 bond of the intact fibrinogen molecule and thrombin proteolysis of the Bβ14-15 bond of Bβ1-42 are listed in Table 1.

Recovery of Bβ1-42 in Blood and Plasma

The recovery of various concentrations of Bβ1-42 (50, 25, 12.5, 6.3, and 3.2 pmol/mL) added to blood and measured directly (employing antiserum R142) was compared with TIFPB determination. The Bβ1-42 assay gave a mean recovery of 114% as compared to 83% with the TIFPB assay.

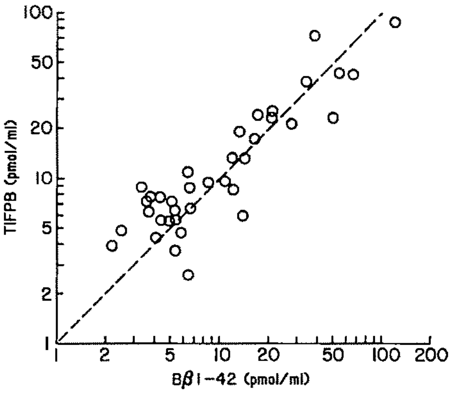

Fig 3. Comparison of plasma Bβ1-42 levels with thrombin increasable fibrinopeptide B immunoreactivity (TIFPB) in 37 patient samples. The coefficient of covariance is 0.91.

Similar results were obtained when Bβ1-42 was added to plasma with a mean recovery of 107% when quantitated as Bβ1-42 and 74% when measured as TIFPB. A comparison of the Bβ1-42 and TIFPB levels in 37 stored patient samples is illustrated in Fig 3. The coefficient of covariance is 0.91.

Bβ1-42 Concentration in Normals

The median Bβ1-42 level in 15 normal individuals was 1.2 pmol/mL with a 10% to 90% range of 1.1 to 1.7 pmol/mL. This correlates favorably with an estimated normal range for TIFPB of 1 to 4 pmol/mL.[11] In contrast, employing antiserum 132, which does not distinguish between Bβ1-42 and β15-42, normal levels of 0.4 pmol/mL were reported.[12] To explore this discrepancy, the stability of Bβ1-42 immunoreactivity in plasma was studied.

Stability of Bβ1-42 Immunoreactivity

Bβ1-42 immunoreactivity was stable in plasma over a 60-minute incubation at 23 °C when measured with antiserum R154 (Fig 4A). In contrast, when antiserum 132 was employed, more than 50% immunoreactivity was lost. Since previous studies had shown loss of fibrinopeptide B immunoreactivity in blood due to cleavage of arginine (Bβ14) by carboxypeptidase-like activity,[22] the effect of porcine pancreas carboxypeptidase B on Bβ1-42 immunoreactivity was determined in a buffer system. Treatment of Bβ1-42 with carboxypeptidase B resulted in a loss of immu-

Table 1. Comparison of Kinetic Constants

Hydrolysis by plasmin	K_m (M)	$K_{cat} \times 10^{10}$ M[(CU/L)s]$^{-1}$	$K_{cat}/K_m \times 10^{6}$ [(CU/L)s]$^{-1}$
Bond hydrolyzed (substrate)			
β42–43 (human fibrinogen)[23]	0.7	4 0	571
β21–22 (Bβ1–42)[13]	18	2.0	11
β14–15 (Bβ1–21)[13]	28	1.2	4
Hydrolysis by thrombin	K_m (M)	$K_{cat} \times 10^{10}$ M[(NIH units/L)s]$^{-1}$	$K_{cat}/K_m \times 10^{6}$ [(NIH units/L)s]$^{-1}$
Bond hydrolyzed (substrate)			
β14–15 (bovine fibrinogen)[35]	11.3	115	1020
β14–15 (human fibrinogen)[36]	16.0	165	1050
β14–15 (Bβ1–42)	28	0.004	0.01

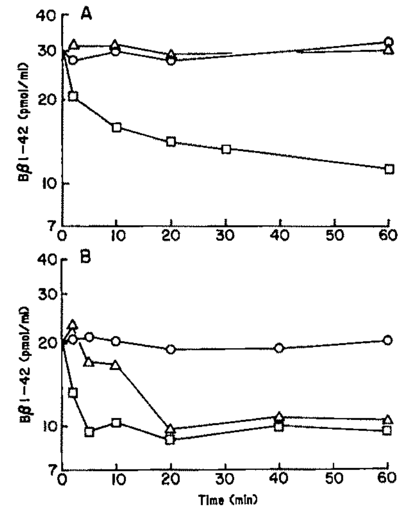

Fig 4. (A) Stability of Bβ1-42 immunoreactivity in plasma: Bβ1-42 (30 pmol/mL) was incubated in plasma at 23 °C. At intervals aliquots were removed, ethanol was added, and Bβ1-42 immunoreactivity was measured with antisera R142 (○), R154 (△), and 132 (□). (B) The effect of carboxypeptidase B on Bβ1-42 immunoreactivity with antiserum 132: Bβ1-42 (20 pmol/mL) was incubated in buffer, pH 7.4, at 23 °C without carboxypeptidase (○) or with 1.25 × 10^{-4} units/pmol (△) or 1.25 × 10^{-3} units/pmol (□) carboxypeptidase B. At intervals, aliquots were removed, o-phenanthroline (.01 mol/L) was added, and residual immunoreactivity measured.

noreactivity similar to that occurring in plasma when the Bβ1-42 was measured with antiserum 132 (Fig 4B). There was no loss of immunoreactivity when antisera R142 or R154 were employed. To determine whether this finding was a result of cleavage of the COOH-terminal arginine (Bβ42), native Bβ1-42 was treated with carboxypeptidase B and the digestion monitored by high performance liquid chromatography. Following 30 minutes of carboxypeptidase treatment (1.0 × 10^{-4} units/pmol Bβ1-42), two major peaks were identified and the corresponding peptides characterized by their amino acid content. One represented residual Bβ1-42 while the other (Table 2) showed loss of an arginine residue and thus was identified as Bβ1-41. Under the same conditions but with a higher carboxypeptidase B concentration, two peptide peaks were again seen. The major peak gave the amino acid analysis shown in Table 2, with loss of an arginine and tyrosine residue and thus was identified as Bβ1-40. The other peak represented a smaller peptide but amino acid analysis was not performed.

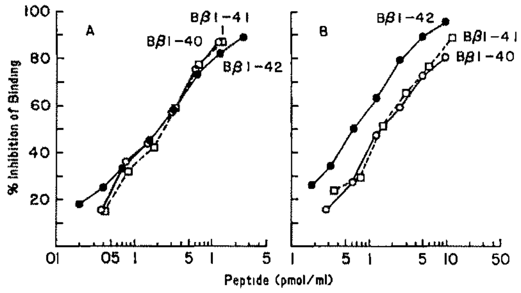

Fig 5. Cross-reactivity of Bβ1-42 and the isolated carboxypeptidase B degradation products. (A) The tracer was ^{125}I-Bβ1-42 and antiserum R142 was used. Inhibition curves for Bβ1-42, Bβ1-41, and Bβ1-40 are shown. 30% tracer was bound. (B) The tracer was ^{125}I-β15-42 and antiserum 132 was used. 32% tracer was bound.

The immunoreactivity of the carboxypeptidase B digestion products was studied using antisera R142 and 132. With antiserum R142 there was complete cross-reactivity between Bβ1-42 and the carboxypeptidase digestion products (Fig 5A). In contrast, when antiserum 132 was employed, Bβ1-41 and Bβ1-40 exhibited a two-fold loss of immunoreactivity as compared to Bβ1-42 (Fig 5B).

Immunochemic Analysis of Plasmin Proteolysis of Fibrinogen

Plasmin cleavage of the Bβ42-43 bond of fibrinogen and subsequent thrombin proteolysis of the Bβ14-15 bond of Bβ1-42 were assessed immunochemically using radioimmu-

Table 2. Amino Acid Analysis of Bβ1-42 After Carboxypeptidase B Digestion

	Bβ1-42 From Sequence Data	Bβ1-42 Before CPB Treatment	CPB-Treated Bβ1-42 After HPLC Separation	
			1 25 × 10^{-4} U/pmol	1 25 × 10^{-3} U/pmol
Aspastic acid	4	4 2	4.2	4.2
Glutamic acid*	5	5 1	5.3	5.1
Proline	6	5.8	6 3	6 1
Glycine	6	5.6	6.3	6 3
Serine	3	2 8	2.9	3 2
Alanine	3	3.0	3 0	3 2
Valine	1	1.1	1.0	1 0
Isoleucine	1	1 1	1 0	1.1
Leucine	2	2 2	2.1	2 3
Phenylalanine	2	2 0	2.1	2.1
Histidine	1	1.0	1 1	1 2
Lysine	2	2.1	1.8	2.0
Tyrosine	1	0.9	0.8	0
Arginine	5	4.9	4.1	4 1

CPB, carboxypeptidase B; HPLC, high performance liquid chromatography.

*One glutamic acid residue represents a pyroglutamic acid residue.

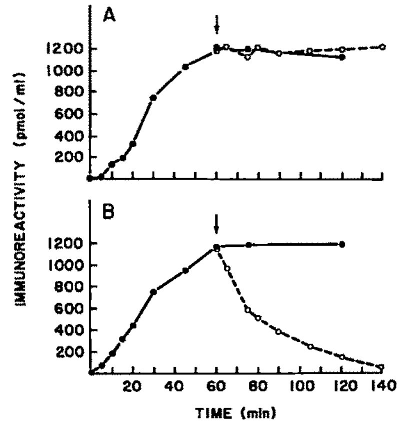

Fig 6. Immunochemical specificity of antisera R142 and R154. Fibrinogen (2.94 μmol/L), urokinase, and plasminogen were incubated at 27 °C. Samples were taken at various time points and immunoreactivity determined using antiserum R154 (A) and R142 (B). At 1 hour thrombin (1.0 u/mL) was added to half the reaction mixture (arrow) and immunoreactivity was monitored with (dashed line) and without (solid line) thrombin addition.

noassays employing antisera R142 and R154 (Fig 6). Proteolysis of fibrinogen by urokinase-activated plasminogen resulted in quantitative release of Bβ1-42 after 60 minutes when either assay was employed. The addition of thrombin to the reaction mixture at this point produced no change in immunoreactivity when determined with antiserum R154 (Fig 6A), whereas progressive loss of immunoreactivity occurred when antiserum R142 was utilized, reflecting thrombin cleavage of Bβ1-42 to FPB and β15-42 (Fig 6B).

Generation of Bβ1-42 in Vitro

When rt-PA was incubated with citrated human plasma, there was progressive generation of Bβ1-42 immunoreactivity (Fig 7A) with concentrations of rt-PA of 50 ng/mL plasma or higher. However, even with the highest concentration of rt-PA employed, this represented <1% of total releasable Bβ1-42 (plasma fibrinogen concentration was 7μmol/L). The plasma FPA concentration of 0.5 pmol/mL did not change throughout the incubation period. The experiment was then performed in plasma pretreated with reptilase (Fig 7B). Here fibrin I, rather than fibrinogen, was the substrate for plasmin action as was confirmed by demonstrating quantitative FPA release within 5 minutes of reptilase addition. Reptilase alone produced no significant Bβ1-42 cleavage but the addition of rt-PA at concentrations of 0.5 and 5 ng/mL, which resulted in no Bβ1-42 release in citrated plasma, produced slow generation of Bβ1-42 for 20 minutes followed by rapid Bβ1-42 release which was still increasing at 60 minutes. With 5 ng rt-PA/mL, less Bβ1-42 was released, but it was still more than that generated by 375 ng/mL in citrated plasma. To determine whether the 20-minute period of slow Bβ1-42 cleavage was due to mechanical limitation of rt-PA access to the fibrin I clot, a subsequent experiment was done in which rt-PA and reptilase were added to plasma simultaneously. This yielded results similar to those shown in Fig 7B.

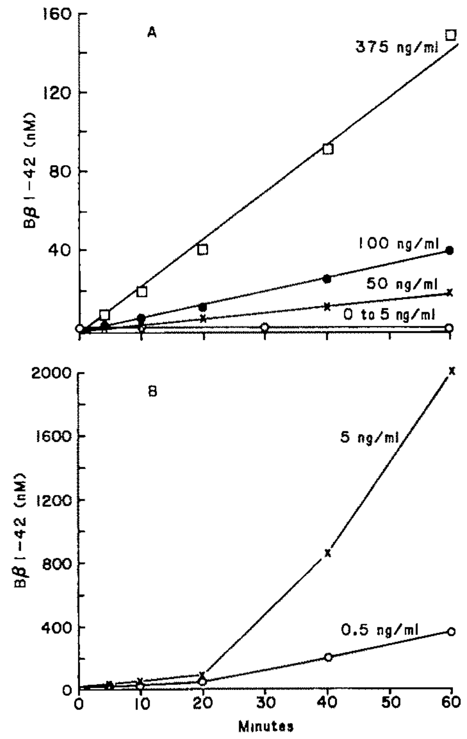

Fig 7. In vitro generation of Bβ1-42. (A) Bβ1-42 cleavage from fibrinogen by rt-PA–mediated plasminogen activation. Recombinant t-PA was incubated with citrated human plasma at 37 °C. At intervals aliquots were removed, mixed with aprotinin (100 U/mL), processed by precipitation with ethanol, and Bβ1-42 immunoreactivity measured with antiserum R142. (B) Bβ1-42 release from a fibrin I clot. The fibrin I clot was first formed by incubation of reptilase with plasma and then rt-PA was added and the mixture incubated at 37 °C. At intervals aliquots were removed, treated as described above, and the Bβ1-42 concentrations measured.

DISCUSSION

Since there is much evidence suggesting that the balance between thrombin and plasmin proteolysis of fibrinogen is an important determinant of normal hemostasis and thrombosis,[1] the development of radioimmunoassays for measurement of specific plasmin-derived fragments from the NH_2-terminal region of the Bβ-chain of fibrinogen permits a direct and quantitative approach to explore this hypothesis. Previous studies of plasmin proteolysis of this region indicated a sequence of events in which Bβ1-42 was the initial cleavage product. Once released from the parent fibrinogen molecule, Bβ1-42 underwent secondary plasmin attack but the kinetic data indicated that these latter cleavages were unlikely to be physiologically important. Whereas Bβ1-42 was the principal species released from fibrinogen or fibrin I by plasmin action, β15-42 was the major fragment cleaved by plasmin proteolysis of fibrin II.[13,23] With the concept that determination of Bβ1-42 levels would reflect plasmin action on fibrinogen or fibrin I while β15-42 levels would reflect proteolysis of fibrin II, we set out to develop specific radioimmunoassays to quantitate these peptides.

Antiserum R142, produced by immunizing with Bβ1-42, has an almost absolute requirement for the presence of an intact Bβ14-15 bond so that peptides lacking this bond are very poor competitors. This is illustrated in Fig 2, where thrombin cleavage of the Bβ14-15 bond of the Bβ1-42 molecule results in progressive loss of Bβ1-42 immunoreactivity which is mirrored by an increase in FPB immunoreactivity. These results are confirmed by HPLC quantitation of the peptides. Thus, the radioimmunoassays for Bβ1-42 and FPB provide an index of plasmin and thrombin activity, respectively, on the NH_2-terminal region of the Bβ-chain of fibrinogen. Recently, Kudryk et al[24] have reported on the preparation and use of a monoclonal antibody to the N-DSK fragment of human fibrinogen that may have an epitope in or around the thrombin-susceptible Bβ14-15 bond such that its specificity appears to be similar to that of antiserum R142.

That Bβ1-42 quantitated in the assay employing antiserum R142 provides a good index of fragment X formation was recently confirmed by a study correlating plasmin-mediated Bβ1-42 cleavage from fibrinogen with peptides released from the COOH-terminal region of the Aα chain. The loss of Aα-chain segments 540–554 and 259–276 coincided with proteolysis of Bβ1-42 measured with this assay. These data indicate that, during fragment X formation, there is simultaneous plasmin-mediated cleavage of the COOH-terminal Aα-chain and NH_2-terminal region of the Bβ-chain of fibrinogen.[25]

Because of the unique specificity of the radioimmunoassay employing antiserum R142, the kinetic data describing thrombin and plasmin proteolysis of Bβ1-42 could be estimated. The stability of Bβ1-42 in the presence of plasmin or thrombin activity in the blood can be explained by these data, which are summarized in Table 1. The Bβ42-43 bond of fibrinogen is the preferential plasmin cleavage site on the NH_2-terminal region of Bβ-chain[3,13,23] with the resultant release of Bβ1-42. Once released, this peptide is a relatively poor substrate for subsequent thrombin or plasmin attack. The selectivity constant (Kse = Kcat/Km) for plasmin cleavage of the Bβ21-22 bond of Bβ1-42 is 140-fold less than that for proteolysis of the Bβ42-43 bond of fibrinogen, suggesting that this hydrolysis is unlikely to be of physiologic importance. Similarly, the selectivity constant for thrombin cleavage of the Bβ14-15 bond of Bβ1-42 is 10^5-fold less than that for proteolysis of the same bond in the intact fibrinogen molecule.

In contrast to the unique specificity of antiserum R142, antiserum R154, obtained by immunizing with β15-42, cross-reacts with several peptides derived from the NH_2-terminal region of the Bβ-chain (Fig 1B). Almost no competition was seen using Bβ1-21, while parallel curves were generated with fragments Bβ1-42, β22-42, and β15-42, suggesting that the epitope for this antiserum is nearer to the COOH-terminal region of the peptide. Thus, its specificity is similar to that of antiserum 132[12] which has been employed in the development of a commercially available radioimmunoassay for quantitating fibrinogen/fibrin fragments containing the β15-42 sequence. Clinical studies utilizing this assay have been reported.[26,27]

The assay employing antiserum R142 gave consistently better recovery of Bβ1-42 added to blood or plasma than the indirect TIFPB assay. However, a comparison of these two methods for quantitation of Bβ1-42 in patient samples yielded a good correlation over a wide range of levels (Fig 3), indicating that TIFPB determination does indeed reflect Bβ1-42, thus validating the results of previous studies which employed this indirect assay.[11]

The discrepancy between the plasma levels of Bβ1-42 in normal individuals as measured in the assay employing antiserum R142 and those reported using antiserum 132,[12] which does not distinguish between β15-42 and Bβ1-42, is best explained by rapid removal of Arg (Bβ42) by plasma carboxypeptidase. This results in at least a 50% loss of Bβ1-42 immunoreactivity when quantitated with the latter antiserum (Fig 4). The finding that the carboxypeptidase digestion products, Bβ1-41 and Bβ1-40, exhibited two-fold less immunoreactivity than Bβ1-42 with antiserum 132 (Fig 5B), indicates that Arg (Bβ42) is an important determinant of the antigenic site. That removal of Tyr (Bβ41), in addition to Arg (Bβ42), resulted in no further loss of immunoreactivity suggests that conformational changes produced by removal of Arg (Bβ42) may be partly responsible for this loss. Although antiserum R154 also cross-reacted completely with Bβ1-42 and β15-42, Bβ1-42 immunoreactivity with this antiserum was stable in plasma and unaffected by in vitro carboxypeptidase treatment. This indicates that the antigenic determinant, although in the COOH-terminal region, is not dependent on Arg (Bβ42). Finally, since the epitope for antiserum R142 is in the region of the Bβ14-15 bond, immunoreactivity measured with this antiserum was unaffected by COOH-terminal degradation of the Bβ1-42 molecule (Fig 5A).

Radioimmunoassays employing antisera R142 and R154, when used in combination, provide a method for quantitating Bβ1-42 and β15-42 release from a variety of substrates. As illustrated in Fig 5, quantitative release of Bβ1-42, mediated by plasmin action on fibrinogen, was obtained using either radioimmunoassay. If thrombin was then added, there was rapid loss of Bβ1-42 immunoreactivity when antiserum R142 was utilized (Fig 5B), reflecting the requirements of this antiserum for an intact Bβ14-15 bond. In contrast, there was no loss of immunoreactivity when antiserum R154 was employed (Fig 5A). Therefore, in terms of plasmin proteolysis of the parent fibrinogen molecule, results obtained utilizing antiserum R142 reflect plasmin activity only on a substrate that has an intact Bβ14-15 bond, ie, fibrinogen or fibrin I. Since antiserum R154 has no such requirement, the result obtained with this assay reflects global plasmin activity regardless of whether the substrate is fibrinogen, fibrin I, or fibrin II.

The in vitro generation of Bβ1-42 was studied by the addition of rt-PA to plasma. The activation of plasminogen by tissue-type plasminogen activator is enhanced in the presence of fibrin.[28-30] The clot-selective properties of this agent have made it an attractive candidate for lytic therapy since it can produce clot lysis without depleting circulating fibrinogen, in contrast to the generalized lytic state produced by streptokinase or urokinase. Specific and quantitative assays for peptides released by plasmin proteolysis of the

NH_2-terminal region of fibrinogen and fibrin provide a method for differentiating the fibrinogenolytic effects of rt-PA from its fibrinolytic activity.

That the Bβ1-42 level is a sensitive index of plasmin-mediated proteolysis of fibrinogen is illustrated by progressive Bβ1-42 generation, without change in the FPA level, on addition of rt-PA to citrated plasma (Fig 7A). With the highest concentration of rt-PA employed there was cleavage of $<1\%$ of the total Bβ1-42 content from the parent fibrinogen molecule, consistent with the clot-selective properties of this agent. However, the finding that Bβ1-42 release was dose-dependent suggests that high plasma concentrations of rt-PA may produce significant fibrinogenolysis. In fact, this has been the reported experience with the administration of rt-PA for the treatment of venous thrombosis.[31] That low doses of rt-PA (0.5 and 5 ng/mL plasma) did not generate Bβ1-42 may be due to the presence of plasma inhibitors to t-PA[32,33] and/or plasmin.

The enhanced plasminogen activation by rt-PA in the presence of fibrin I is demonstrated by the significant Bβ1-42 release in reptilase-treated plasma with concentrations of rt-PA which had no effect in citrated plasma (Fig 7B). The period of slow Bβ1-42 release over the initial 20 minutes is unlikely to be due to mechanical limitation of rt-PA access into the fibrin clot since the simultaneous addition of rt-PA and reptilase produced similar results. A possible explanation relates to the finding that tissue plasminogen activator can induce plasminogen binding to fibrin I by catalyzing plasmin-mediated modification of the fibrin substrate and exposing new binding sites for Glu-plasminogen.[34]

In summary then, radioimmunoassays have been developed that can quantitate the products of plasmin cleavage of the NH_2-terminal region of the Bβ-chain of fibrinogen. The kinetic studies employing the specific Bβ1-42 assay explain the stability of Bβ1-42 in plasma, thus confirming the utility of measurement of this peptide. Because of carboxypeptidase-mediated degradation, the specificity of antisera to Bβ1-42 must be extensively explored prior to development of clinical assays for this peptide. Antiserum R154, because of its cross-reactivity with the carboxypeptidase-mediated degradation products, may prove useful in the development of a radioimmunoassay for global plasmin activity. Assays employing these antisera provide a direct and quantitative method to further explore the hypothesis that the balance between thrombin and plasmin proteolysis of fibrinogen is an important determinant of normal hemostasis and thrombosis. Further, they may prove useful in monitoring patients undergoing thrombolytic therapy.

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Assessing the Delivery of Neutrophils to Tissues in Neutropenia

By D.G. Wright, A.I. Meierovics, and J.M. Foxley

Studies of neutrophil kinetics in neutropenic individuals, as well as clinical observations of variability in the occurrence of infection among patients with neutropenia, have suggested that blood neutrophil counts may not uniformly reflect the effective delivery of neutrophils to extravascular tissues where the cells perform their principal host defense functions. To evaluate this possibility we developed a sensitive, reproducible method of measuring the extravascular delivery of neutrophils to a normal mucosal site of neutrophil turnover. This method is based upon the quantification of neutrophils recoverable from saline mouth wash specimens. Twenty-five mL specimens, obtained in a controlled manner from neutropenic patients and normal subjects, were centrifuged and the sediments resuspended in 1.0 mL Hank's buffer with 2 μg acridine orange, incubated at 37 °C for 15 minutes, and then examined in a hemocytometer chamber by fluorescence microscopy. Neutrophils could be clearly distinguished by their characteristic fluorescence and were counted. With this method as few as 1,500 neutrophils were detected reliably in mouth wash specimens. Mucosal neutrophil counts varied less than 10% with repeated sampling of individual subjects over 5-day periods and were consistently greater than 1.3 × 10^5/specimen in non-neutropenic individuals. Although profound neutropenia was generally reflected by lower than normal oral mucosal neutrophil counts, these counts were significantly higher in individuals with chronic severe neutropenia (blood neutrophils <300/mm^3) than in patients with acute neutropenia of comparable severity that had developed following chemotherapy. Also, in individuals recovering from profound neutropenia, neutrophils usually reappeared earlier in mouth wash specimens than in blood, and oral mucosal neutrophil counts attained recovery levels more rapidly than did blood counts. This phenomenon was particularly evident in an individual with cyclic neutropenia. Moreover, mucosal neutrophils could occasionally be detected in profoundly neutropenic patients when neutrophils were not present in blood samples. These findings indicate that mucosal neutrophil counts in individuals with neutropenia provide information about the delivery of neutrophils to tissues that may not be apparent from blood neutrophil counts alone.

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IT IS well known that neutropenia is associated with an increased risk of bacterial and fungal infections, particularly when blood neutrophil counts are less than 500/mm^3. However, neutropenic individuals may vary considerably in their apparent susceptibility to infection. The incidence and severity of infections are especially variable among individuals with chronic neutropenias. The clinical courses of such individuals are often remarkably benign despite profound neutropenia, unlike the usual experience of patients with an equivalent degree of neutropenia that is of acute onset following myelotoxic chemotherapy or radiation.[1-3]

Variability in the susceptibility to infection in neutropenia suggests that blood neutrophil counts may not uniformly reflect either the body's supply of neutrophils or the delivery of neutrophils to extravascular tissue sites where the cells perform their principal host defense functions. Furthermore, studies of neutrophil kinetics in both neutropenic and normal individuals, using techniques dependent upon the measurement of radiolabeled neutrophils in the circulation, have shown that an acute or chronic redistribution of neutrophils from marrow storage to the intravascular space and/or between circulating and marginated pools may cause variations in blood neutrophil counts that do not reflect changes in the body's total neutrophil supplies or the neutrophil turnover rate.[4-8] It has been observed in particular that the proportion of neutrophils in the marginated pool tends to increase as blood neutrophil concentrations decrease both in normal subjects[4] and in neutropenic patients.[6] These observations also suggest that blood neutrophil counts by themselves may be misleading with respect to the actual availability of neutrophils to tissues in neutropenic individuals. Studies of the delivery of neutrophils to tissues using the Rebuck skin window technique have in general demonstrated an impairment of neutrophil delivery to tissues in profoundly neutropenic patients.[1,9,10] However, this technique, which is time consuming and difficult to standardize, is unlikely to be able to detect differences in the delivery of neutrophils to tissues that may exist among individuals with similar degrees of neutropenia, nor is it readily applicable to the study of patients with profound neutropenia associated with myelosuppression and thrombocytopenia.

In order to examine variations in the delivery of neutrophils to extravascular tissues that may exist in neutropenia, we developed a method of quantifying neutrophils that can be washed from the oral mucosa. This method is a refined version of a technique that has been used for some time to provide a semiquantitative measure of periodontal disease severity in hematologically normal individuals.[11,12] Using this method, we find that mucosal neutrophil counts provide information about the delivery of neutrophils to tissues that may not be apparent from blood neutrophil counts alone. We find, in particular, that the supply of neutrophils to tissues as reflected by mucosal neutrophil counts is frequently underestimated by blood neutrophil counts in patients with chronic neutropenias and during the early phases of recovery from acute neutropenic episodes.

MATERIALS AND METHODS

Study subjects. Twenty-seven healthy men and women between the ages of 22 and 58, with a normal oral and pharyngeal exam and with teeth in good repair, were studied to define a normal range for measurements of oral mucosal neutrophil counts. Mucosal neutrophil counts were also measured in two men and two women (ages 51 to 60) who were edentulous but otherwise in good health. These

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healthy individuals were specifically without clinically evident oral mucosal disease or symptoms, and all had normal blood neutrophil counts (2000 to 6000/mm^3) at the time of study.

Twenty-six individuals with either acute or chronic neutropenia, who were patients at the Walter Reed Army Hospital, were also studied. Of these, eight had only mild to moderate degrees of neutropenia (between 500 and 1800/mm^3) at the time of study and 17 had blood neutrophil counts in the severely neutropenic range (0 to 300/mm^3). Acute neutropenias were in all cases the result of myelosuppressive chemotherapy given within seven days of the onset of neutropenia. All patients with chronic neutropenias were known to have had neutropenia for at least 2 months prior to study. Underlying conditions in these neutropenic subjects are summarized in Table 1.

Patients and healthy volunteers were intentionally selected such that the study populations would be free of moderate or severe oral mucosal disease. The oral mucosa was examined in potential study subjects and the status of mucosal tissues was estimated by defining a gingival index.[9] The scoring criteria for determining this gingival index were as follows: a score of 0 = an absence of inflammation in gingival tissues; 1 = mild inflammation, characterized by reddening of the tissues without change in texture; 2 = moderate inflammation, characterized by moderate glazing, redness, edema, and hypertrophy of gingival tissues with or without bleeding on pressure from a probe; 3 = severe inflammation, characterized by marked redness and hypertrophy with a tendency toward spontaneous bleeding, with or without ulcerations. All of the normal subjects had a gingival index score of 0, and none of the neutropenic subjects included in this study (other than a patient with cyclic neutropenia) had a gingival index score greater than 1 at the times that mucosal neutrophil counts were measured. All study subjects gave informed consent for the examinations that were performed in accordance with a human use protocol approved at Walter Reed.

Recovery and quantification of neutrophils from the oral mucosa. Neutrophils present in the tissues of the oral mucosa were quantified by counting these cells in timed, saline mouth wash specimens. After an initial evaluation of several variations in technique discussed below, the following basic method for obtaining and examining specimens was used. Sterile, normal saline solution (0.9 g%) was measured into 25 mL aliquots in sputum cups. Study subjects swirled these measured amounts of saline in their mouths for 30 seconds, timed by a stop watch, and then returned the specimen to the sputum cup. Duplicate specimens were obtained with each sampling, one specimen obtained immediately following the other. The specimens were then centrifuged at 200 g for 10 minutes (within one hour of sampling), the supernatants decanted and discarded, and the pellets resuspended with a pasteur pipette in 1.0 mL of Hank's balanced salt solution (without phenol red) which contained 2.0 μg/mL acridine orange (3,6-bis[Dimethylamino] acridium chloride hemi-[zinc chloride salt], Sigma, St. Louis, Mo). These sediment suspensions were then incubated for 15 minutes at 37 °C in a shaking water bath and again resuspended thoroughly with a pasteur pipette. A drop of each suspension was transferred to a hemocytometer chamber (American Optical, Buffalo, NY) and examined by fluorescence microscopy, using a 20 x, long focal length objective lens and 10 x ocular lenses.

Neutrophils were clearly distinguishable from extraneous cellular and microbial debris in the specimen by their characteristic orange granular fluorescence (Fig 1B). Rarely, budding yeast forms or epithelial cells showed some orange fluorescence but this was readily differentiated from that of neutrophils in the specimen. Monocytes were also observed to fluoresce after staining with acridine orange. However, the orange granular fluorescence and yellow-green nuclear fluorescence of monocytes is much less bright than that of neutrophils, and these cells are distinguishable both by cytofluorography[13] and by eye. In performing mucosal neutrophil counts, cells with equivocal fluorescence staining were not counted.

Table 1. Neutropenic Subjects: Underlying Conditions

Acute Neutropenia Following Cytotoxic Chemotherapy		Chronic Neutropenia	
Acute myelogenous leukemia	4	Idiopathic	5
Acute lymphocytic leukemia	2	Felty's Syndrome	4
Non-Hodgkins lymphoma	4	Associated with CLL	2
Metastatic carcinoma of breast	3	Cyclic neutropenia	1
Metastatic carcinoma of bowel	1		
Total	14		12

The area that the 20 x microscope field covered on the hemocytometer grid (final magnification = 200 x) was determined to be 0.672 mm^2, defining a volume of 0.672 mm^3 per 10 fields. Total neutrophils seen in 10 separate microscope fields were counted, and the numbers converted to the neutrophil concentration in the mouth wash sediment suspension according to the following formula: Neutrophils in specimen = (number of cells/10 fields) × (1.49) × (10^3). For convenience, mucosal neutrophil counts were expressed in general as cells/10 microscope fields. In serial studies with patients, saline mouth wash specimens were always obtained at mid-morning (about 10 AM).

For certain studies, up to six successive mouth wash specimens were obtained, one immediately after the other; replicate specimens were also obtained at 15-minute intervals. To assess the accuracy of this fluorescence staining method for counting neutrophils, the method was also applied to counting neutrophils present in leukocyte suspensions prepared by dextran sedimentation of normal venous blood, for which the numbers of neutrophils, monocytes, and lymphocytes had been calculated separately from standard chamber counts and differentials of Wright-Giemsa stained cytopreps.

Peripheral blood neutrophil counts. For blood neutrophil counts, venous blood samples (5 mL) were collected into standard vacutainer tubes anticoagulated with EDTA (Vacutainer Systems, Rutherford, NJ). Blood smears stained by an automated method with Wright-Giemsa were prepared, and total white blood cell counts were determined by a Coulter particle counter (Coulter Electronics, Hialeah, Fla). Absolute neutrophil counts were calculated from total white blood cell counts and 200 cell leukocyte differentials done exclusively by one of two observers (DW or JF). In serial studies with patients and with normal volunteers, blood samples were routinely obtained shortly before the mouth wash specimens.

RESULTS

Recovery of oral mucosal neutrophils from healthy hematologically normal individuals. Neutrophils could be readily seen in Wright-Giemsa stained, cytospin preparations of oral saline rinses obtained from hematologically normal, healthy adults who were free of oral or dental disease (Fig 1A). However, in order to quantify these cells in a reproducible manner and with some degree of detection sensitivity, it was necessary to use a selective staining technique that would permit relatively small numbers of neutrophils to be clearly distinguished from a large and highly variable quantity of epithelial cells, cellular debris, and microbial flora. This was accomplished by exploiting the fluorescence staining properties of acridine orange, a stain upon which certain automated fluorescence activated leukocyte differential systems have been based.[13] As is illustrated in Fig 1B, neutrophils that have taken up this stain fluoresce

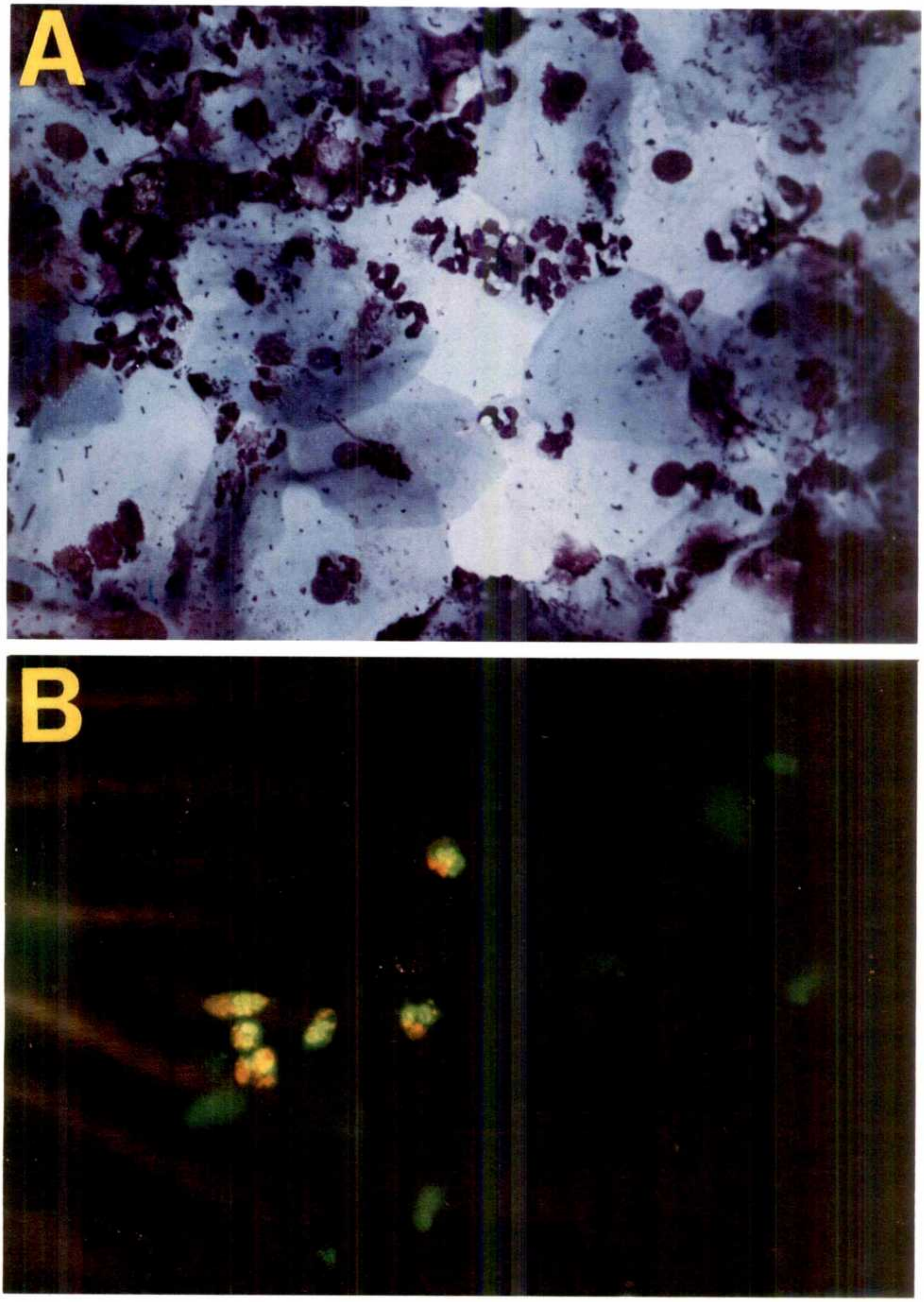

Fig 1. Oral mucosal neutrophils. (A) Wright-Giemsa stained, cytospin preparation of a saline mouth rinse specimen from a hematologically normal volunteer with healthy oral mucosa (approximate magnification × 300). (B) Fluorescence microscopy of acridine orange stained suspension of mouth rinse sediment from healthy normal volunteer (approximate magnification × 300).

brightly and stand out clearly under fluorescence microscopy. Because of their characteristic orange granular fluorescence, nuclear morphology, and size, neutrophils could be recognized and counted, even if present in very low numbers. By combining this staining technique with a standard hemocytometer chamber, it was possible to measure neutrophil concentrations reproducibly down to a detection threshold of approximately 1,500 neutrophils in an individual 25 mL mouthwash specimen. This fluorescence staining method was found to detect neutrophils accurately in a mixed leukocyte preparation over a broad range of cell concentrations (Fig 2).

Counts from two successive mouthwash specimens, one obtained immediately after the other, were averaged. Unexpectedly, the neutrophil count in the second of these successive specimens was not much lower on average than that in the first specimen (2nd specimen count = 92 ± 7% of 1st specimen count, mean ± SEM, n = 32), although neutrophil counts dropped markedly in subsequent mouth wash specimens obtained immediately thereafter (Fig 3A). Moreover, the mean values for two successive counts showed less variability than either count alone when normal volunteers were studied repeatedly at 1-day intervals. Addition of EDTA (10 mg %) to the saline rinse solution increased the recovery of neutrophils slightly (by 5% to 15%) but did not provide more reproducible mucosal neutrophil counts than did normal saline alone, which was more readily applicable to studies of neutropenic patients.

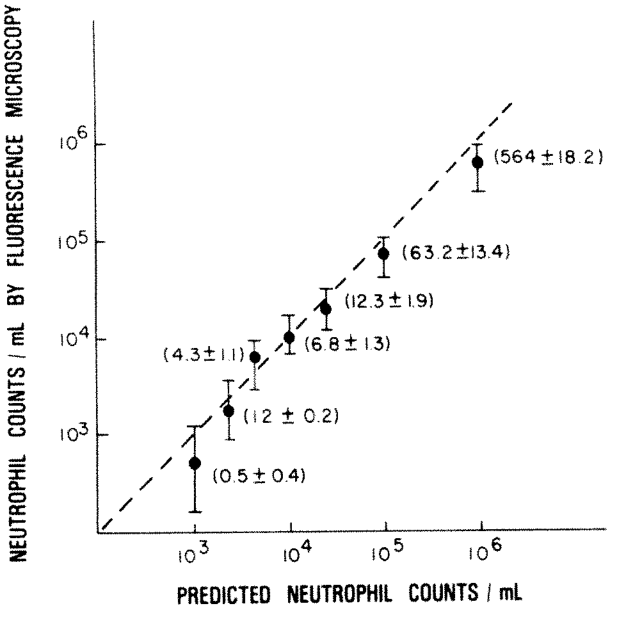

Fig 2. Neutrophil counts obtained by fluorescence microscopy of acridine orange stained suspensions of leukocytes (isolated from venous blood by dextran sedimentation) plotted against predicted counts. Samples for counting were prepared by multiple dilutions of a cell suspension with known neutrophil concentration (10^7/mL). This leukocyte suspension contained 75% neutrophils, 8% monocytes, and 17% lymphocytes. Numbers in parentheses represent neutrophils/10 microscope fields detected by fluorescence microscopy. Means ± SEM for 5 replicate determinations are shown.

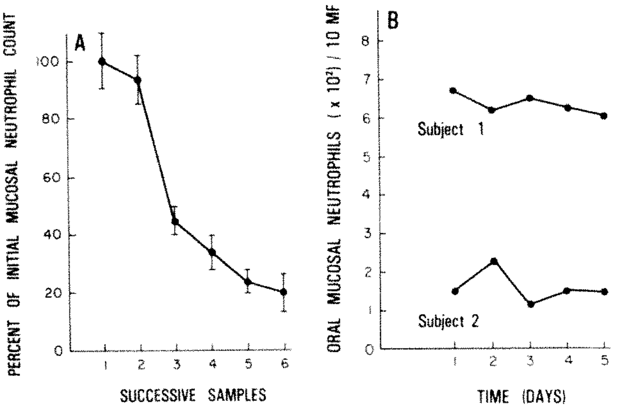

Fig 3. Repetitive oral mucosal neutrophil counts in normal individuals. (A) Mucosal neutrophil counts in successive mouth wash specimens, one taken immediately after the other. Results represent means ± SEM from 5 replicate studies. (B) Daily mucosal neutrophil counts in two healthy subjects whose blood neutrophil counts averaged 3,825 ± 219/mm³ (subject 1) and 2,670 ± 337 (subject 2).

Mouthwash samples from normal volunteers were found to contain up to 10.8 × 10^5 neutrophils and never less than 1.3 × 10^5/sample unless the individual was edentulous (see Fig 4A). Even in this group of study subjects with normal gingival indices and equivalent dentition, there was considerable variability in mucosal neutrophil counts from person to person, and only a very loose correlation between blood neutrophil counts and mucosal neutrophil counts (Fig 4A). However, there was much less variability in mucosal neutro-

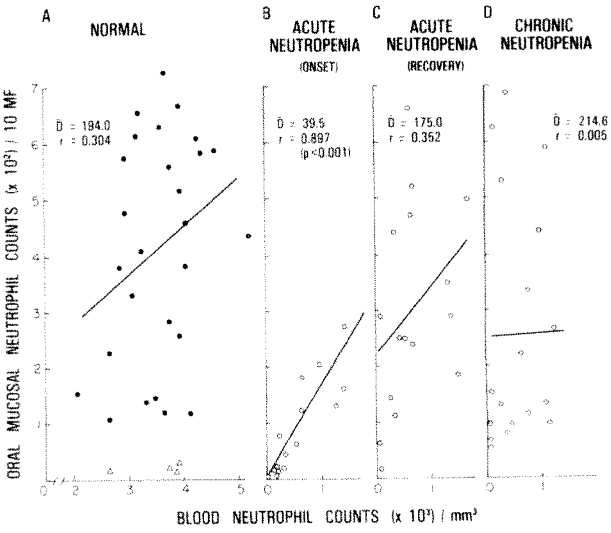

Fig 4. (A) Oral mucosal neutrophil counts in 31 healthy, nonneutropenic adults, 4 of whom (triangles) were edentulous. (B and C) In 5 patients with acute neutropenia during onset of neutropenia (3 data points from each patient), and during recovery from neutropenia (3 data points from each of 4 patients, 4 points from 1 patient). (D) In 8 patients with chronic neutropenia (2 data points from each of 6 patients and 3 points from each of 2 patients). Mucosal neutrophil counts are plotted against blood neutrophil counts obtained at the same time. Linear regression plots, average deviations from linearity (D̄), and correlation coefficients (r) are shown. MF = microscope fields (× 200).

phil counts obtained repeatedly from a single person on successive days. This variability was on average less than 10% for 6 normal subjects when studied on each of 5 successive days, or at intervals of greater than 2 hours. Representative results from these studies are shown in Fig 3B.

When mucosal neutrophil counts were obtained from normal subjects at 15-minute intervals (each count representing the average from two successive mouth wash specimens taken at each interval), it was found that the counts fell to a relatively stable level by the third set of samples. Pooled data from 5 normal subjects studied in this manner are shown in Fig 5. If one assumes that the reduced but stable level of mucosal neutrophil counts observed with successive mouth wash sampling represents a balance between the loss of cells that are washed from the mucosa and the arrival of new cells, then this level may be considered to reflect the rate at which neutrophils are delivered to the mucosal sites that are sampled.

Corticosteroids when taken daily have been shown to cause an impairment in the delivery of neutrophils to tissues, as measured by the Rebuck skin window assay.[14] This effect of corticosteroids could also be detected with mucosal neutrophil counts. In studies of healthy volunteers, it was found that mucosal neutrophil counts were significantly reduced 6 hours after a single dose of 40 mg prednisone (by 46 ± 12%, mean ± SEM from five replicate studies) even though blood neutrophil counts had more than doubled. As shown in Fig 5, the apparent rate of neutrophil delivery to the oral mucosa reflected by repeat counts at 15-minute intervals was also reduced by this corticosteroid.

Recovery of mucosal neutrophils from neutropenic individuals. Profound neutropenia (< 300/mm^3) was in general associated with decreased mucosal neutrophil counts compared with normal controls (Figs 4 B, C, D). However, as with normal individuals, there was considerable variability in mucosal neutrophil counts among individuals with comparable degrees of neutropenia, particularly in the mild to moderately neutropenic range (500 to 1800/mm^3). As shown in Figs 4 B, C, and D, the highest degree of correlation between blood neutrophil counts and mucosal neutrophil counts was observed in patients with acute neutropenia during the onset of neutropenia; such correlations were much looser in these same patients during recovery from neutropenia and were inapparent in data from patients with chronic neutropenias.

In patients with acute, severe neutropenia following myelosuppressive chemotherapy, neutrophils were commonly observed to reappear and return to a stable level earlier in the oral mucosa than in the blood. This phenomenon is illustrated in Fig 6A. An analogous phenomenon was very obvious in serial counts obtained from a young woman with cyclic neutropenia (Fig 7).

It was also observed that low but detectable numbers of neutrophils could be recovered occasionally from the oral mucosa in patients with no neutrophils evident in the blood on repeated sampling. An example of this is shown in Fig 6B, which presents data obtained from a patient who was observed for nine days to have no detectable neutrophils in the blood, and who then developed worsening clinical signs of

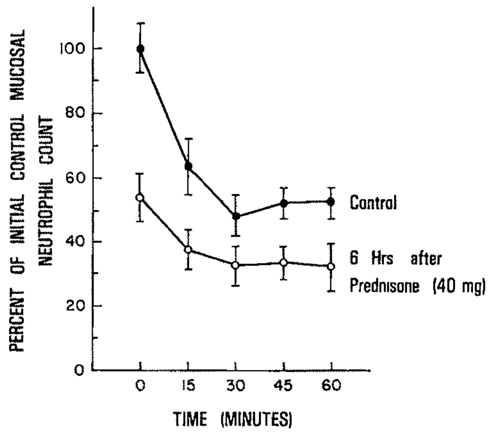

Fig 5. Mucosal neutrophil counts obtained at 15-minute intervals in healthy adults before (closed circles) and 6 hours after 40 mg prednisone P.O. (open circles). Blood neutrophil counts in these individuals were 268 ± 51% higher after prednisone. Results represent means ± SEM from 5 studies.

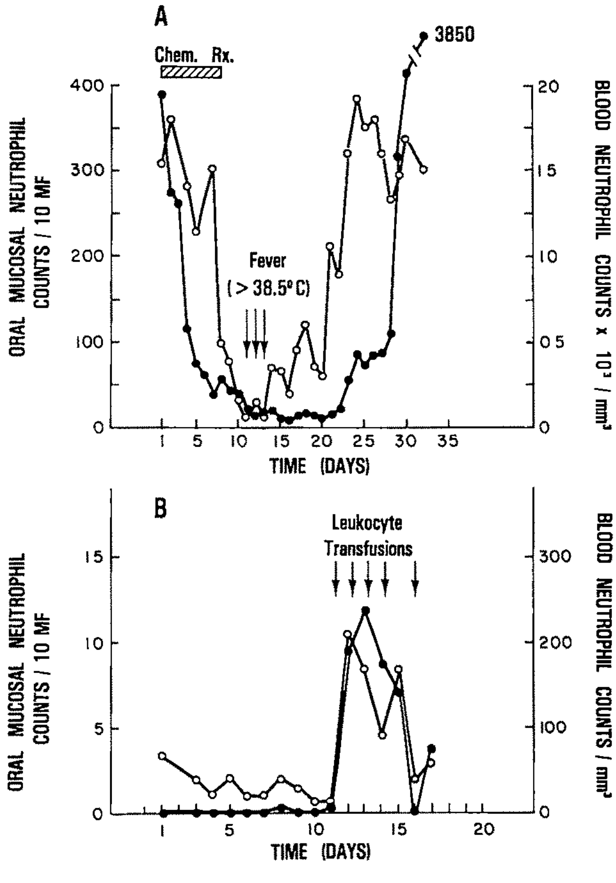

Fig 6. Serial blood and oral mucosal neutrophil counts in individuals with profound neutropenia. (A) 41-year-old woman with AML in remission, during and following a course of consolidation chemotherapy. (B) 35-year-old woman with AML in relapse following multiple courses of chemotherapy for reinduction of remission. Closed circles, solid lines = blood neutrophil counts; open circles, dashed lines = oral mucosal neutrophil counts.

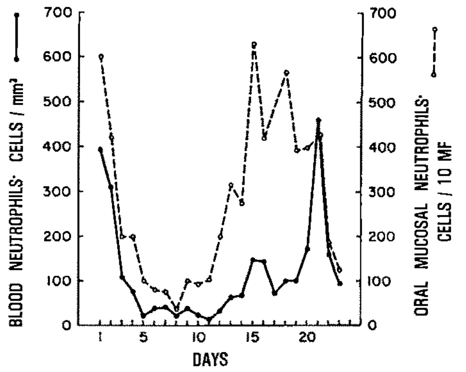

Fig 7. Serial blood and oral mucosal neutrophil counts in a 21-year-old woman with cyclic neutropenia. Closed circles, solid lines = blood neutrophil counts; open circles, dashed lines = oral mucosal neutrophil counts.

infection, despite broad spectrum antibiotics, prompting the institution of leukocyte transfusions. Of interest, it appeared that the effects of leukocyte transfusion could be detected in mucosal neutrophil counts in this patient Although it was not possible to prove in this case that the rise in blood and mucosal neutrophil counts observed with leukocyte transfusions reflected the presence of donor cells, this was highly likely since blood neutrophil counts returned to zero after a day's hiatus in leukocyte transfusions.

Patients with profound degrees of neutropenia (<300/mm^3) that was chronic (ie, established for ≥ 2 months) were distinguishable as a group from patients with acute neutropenias of comparable degrees of severity by the oral mucosal neutrophil counts. As shown in Table 2, mucosal neutrophil counts from patients with profound chronic neutropenias (0 to 100/mm^3 and 100 to 300/mm^3) were significantly higher than those from patients with acute neutropenias of comparable severity. It was possible that differences in the status of the oral mucosa in these two groups of patients contributed to this observation; however, such differences were not evident from clinical examination of the oral mucosa.

The relevance of mucosal neutrophil counts to the clinical status of these markedly neutropenic patients was suggested by relationships that appeared to exist between the blood and mucosal neutrophil counts and the presence or absence of fever. None of the patients with chronic neutropenias, whose blood and mucosal neutrophil counts are summarized in Table 2, were febrile when studied. In contrast, most of the acutely neutropenic patients represented in Table 2 were febrile at some time during their nadirs of neutropenia.

As has been pointed out above, one of the distinctions between these groups of patients was that mucosal neutrophil counts were on average substantially higher with respect to blood neutrophil counts for patients with chronic, profound neutropenias than was the case for patients who were acutely neutropenic. Moreover, when the relationship of mucosal neutrophil counts to blood neutrophil counts was expressed as a ratio (mucosal neutrophils per 10 microscope fields, divided by blood neutrophils per mm^3), it was found that this ratio was almost always > 1.0 for patients with chronic neutropenias, while it was frequently < 1.0 for the acutely neutropenic patients. Moreover, when individual patients were followed throughout episodes of myelosuppression following cytotoxic chemotherapy, it was observed that the highest degrees of fever tended to occur when the ratios of mucosal neutrophil counts to blood neutrophil counts were lowest, as exemplified by the data shown in Fig 6A.

Table 3 summarizes data from five patients who were studied repeatedly during chemotherapy-induced acute neutropenia. Mucosal and blood neutrophil counts were obtained from each of these patients on at least six separate days during their nadirs of neutropenia. These patients were febrile (≥ 38 °C) on 16 of 30 occasions (53%) when their neutrophil counts were studied; fever ≥ 38.5 °C was recorded on six occasions (20%). As might be expected, the highest fevers tended to occur more frequently with blood neutrophil counts of 0 to 100/mm^3 than with counts of 100 to 300/mm^3 ($P = 0.10$, Chi Square; $P = 0.18$, Fisher exact test). However, when these same data were examined with respect to the ratios of mucosal to blood neutrophil counts, it was found that fever (≥ 38.5 °C) was significantly more likely to be present when the mucosal/blood neutrophil ratio was ≤ 0.4 (the median value observed in these patients), both when all observations (blood neutrophils = 0 to 300/mm^3) were analyzed ($P < 0.01$, Chi Square; $P < 0.02$, Fisher exact test) and when subgroups of observations (blood neutrophils = 0 to 100/mm^3) were analyzed ($P < 0.05$, Fisher exact test).

DISCUSSION

It has long been recognized that neutrophils are normally present on mucosal surfaces, in saliva, and in tears. According to an old clinical adage, a patient's awareness of a dried conjunctival exudate at the corner of the eye upon awakening is a very early sign of recovery from agranulocytosis. Furthermore, it has been suggested that the presence of neutrophils in conjuctival secretions, or in a swab of the nasal mucosa, might provide useful information for assessing the relative risk of infection in a patient with profound neutropenia.[15] Animal studies have indicated that the delivery of neutrophils to mucosal tissues, particularly that of the gastrointestinal tract, accounts for a substantial proportion of daily neutrophil turnover,[16] and it would appear likely that a constant turnover of neutrophils at such sites constitutes an important component of mucosal barriers against the invasion of resident microbial flora, for when production and turnover of neutrophils cease, sepsis with organisms from this normal flora quickly ensues.

For the studies described above, we developed a method for quantifying mucosal neutrophils recoverable in mouth wash specimens. We then used this method to determine if mucosal neutrophil counts might provide information that is different from or supplemental to that provided by blood neutrophil counts in neutropenic individuals. There are a number of reasons to suspect that the measurement of neutrophils that have been delivered to a normal tissue site of turnover in neutropenic individuals might differ from that of blood neutrophils, which are in transit to tissues. First, if neutropenia is associated with substantial shifts in the proportion of neutrophils in the circulating pool to that of the

Table 2. Relationship Between Blood Neutrophil Counts and Oral Mucosal Neutrophil Counts in Patients With Severe Neutropenia

Range of Blood Neutrophil Counts (PMNS/mm^3)	Acute Neutropenia*		Chronic Neutropenia†	
	Blood (PMNS/mm^3)‡	Mucosa (PMNS/10 MF)§	Blood (PMNS/mm^3)	Mucosa (PMNS/10 MF)
0–100	56 ± 8 (10)	31 ± 9	61 ± 11 (7)	179 ± 47
100–300	182 ± 14 (12)	34 ± 10	185 ± 21 (10)	452 ± 69

*Observations from 9 patients with acute neutropenia following cytotoxic chemotherapy (3 AML, 2 ALL, 2 Non-Hodgkins lymphoma, 2 Ca-Breast). Data represent the lowest blood neutrophil counts observed in these patients (3 observations from each of 6 patients, 2 observations from 1 patient, and 1 observation from 2 patients). Mean gingival index scores at times of study for patients with blood neutrophil counts of 0–100/mm^3 = 0.7, and for patients with blood neutrophil counts of 100–300/mm^3 = 0 8.

†Observations from 8 patients with chronic neutropenia syndromes (2 Idiopathic, 4 Felty's, 2 CLL). Two observations are from each of 7 patients and 3 observations are from 1 patient. Mean gingival index scores at times of study for patients with blood neutrophil counts of 0–100/mm^3 = 0.8, and for patients with blood neutrophil counts of 100–300/mm^3 = 0.8.

‡Results expressed as mean values ± SEM (number of observations).

§MF = microscope fields.

marginated pool, as has been suggested by neutrophil kinetic studies to be the case in neutropenia,[4,6] or if neutropenia is associated with a decrease in the transit time of neutrophils through the blood to tissues, then one can expect that blood neutrophil counts might underestimate the supplies of neutrophils available to tissues. Second, clinical observations of variability in the occurrence of infection among patients with acute and chronic neutropenias suggest that blood neutrophil counts may not uniformly reflect the effective delivery of neutrophils to extravascular tissues where the cells perform their principal host defense functions.

The results of our studies indicate that blood neutrophil counts do indeed underestimate the delivery of neutrophils to tissues in certain settings of profound neutropenia, particularly among individuals with chronic, steady-state neutropenias, and during early recovery from an episode of myelosuppression when neutrophil reserves and production are returning. An apparent discordance of blood neutrophil counts with the delivery of neutrophils to tissues was also detected by mucosal neutrophil counts in normal individuals who had taken corticosteroid. This finding is consistent with previous observations from studies using the Rebuck skin window technique.[14]

As would be expected, mucosal neutrophil counts indicated in general that the delivery of neutrophils to tissues is impaired in profound neutropenia. Moreover, a direct relationship between blood neutrophil counts and mucosal neutrophil counts was found to be particularly strong during the development of neutropenia following myelosuppression. This relationship, however, was much less apparent during recovery from myelosuppression and among chronically neutropenic patients in whom neutrophil production is preserved albeit at a limited level. The finding that neutrophils reappeared earlier in mucosal counts than in blood counts during myelopoietic recovery is consistent with the diminished correlation between blood and mucosal neutrophil counts that was observed during this phase of acute neutropenia.

Approximations of the relative numbers of neutrophils in mouth rinse specimens have been used to grade the severity of periodontal disease.[11,18] Nonetheless, the presence of neutrophils in saliva or on the oral mucosa is not per se an indication of mucosal pathology or oral infection, for these cells can be readily detected in healthy individuals with normal mucosal and gingival tissues and with normal dentition. The principal sites of the oral mucosa for neutrophil turnover are in alveolar crevices surrounding the teeth,[18] and our finding of relatively low numbers of oral mucosal neutrophils in edentulous but otherwise healthy people is entirely consistent with the prior observations of others.[18,19] Variations in the numbers of teeth were, however, unlikely to account for the variability in mucosal neutrophil counts observed among normal and neutropenic individuals, for all study subjects were selected for normal dentition and differed only with respect to the numbers of wisdom molars that were missing.

Counts of oral mucosal neutrophils have been studied

Table 3. Fever in Patients With Acute Neutropenia*

		Blood PMNs = 0–100/mm^3	Blood PMNs = 100–300/mm^3
Fever ≥38 °C	M/B† > 0.4	29% (2/7)‡	38% (3/8)
	M/B ≤ 0.4	78% (7/9)	67% (4/6)
	Total	56% (9/16)	50% (7/14)
Fever ≥38.5 °C	M/B > 0.4	0% (0/7)§	0% (0/8)
	M/B ≤ 0 4	56% (5/9)	17% (1/6)
	Total	31% (5/16)	7% (1/14)

*Observations from 5 patients during the nadir of neutropenia following cytotoxic chemotherapy; 6 observations from each patient.

†M/B = mucosal neutrophil count (PMNs/10 MF) divided by the blood neutrophil count (PMNs/mm^3).

‡Percent of observations associated with fever (number with fever/total number).

§Interrelationship between mucosal/blood neutrophil counts and the presence or absence of fever is significant by Fisher exact test ($P < 0.05$).

previously as a means of assessing the effects of leukocyte transfusions[20]; however, the techniques used in these prior studies were not of sufficient sensitivity to detect either endogenous or transfused neutrophils in profoundly neutropenic individuals except when clinically evident oral mucositis was present. The techniques developed for our present studies, on the other hand, proved to be sufficiently sensitive to detect the presence of mucosal neutrophils even, in some cases, when neutrophils were undetectable in the blood.

Differences in the status of the oral mucosa could account in part for differences in the numbers of oral mucosal neutrophils that were observed among patients with acute and chronic neutropenias, for it is well known that individuals with chronic neutropenias tend to develop recurrent stomatitis and periodontal disease, as was the case in the patient with cyclic neutropenia described in this report. However, if there were differences in the oral mucosa among our study subjects, they were not apparent by direct inspection alone. These subjects were intentionally selected to be free of gingival and dental disease in order to eliminate mucosal inflammation as an independent variable as much as possible.

In any case, our observations are of interest because individuals with chronic, established neutropenias, even when profound, tend to present a relatively benign clinical picture compared with patients who develop acute neutropenia following myelosuppressive chemotherapy.[3] Patients with chronic neutropenias rarely develop sudden, overwhelming sepsis or septicemias,[1,2] and this clinical distinction is consistent with a difference in the effective delivery of neutrophils to normal mucosal sites of neutrophil turnover.

It has been suggested that monocytes, which are often preserved in chronic neutropenias and may be increased in number, may assume some of the host defense functions of neutrophils, thereby accounting for the relatively mild host defense defect that is often apparent with chronic neutropenia.[10] Monocytes could possibly have masqueraded as neutrophils in the mucosal counts performed on chronically neutropenic study subjects. However, it is unlikely that monocytes accounted for much, if any, of the mucosal neutrophil numbers detected in these patients since the fluorescence staining technique used distinguishes neutrophils from monocytes fairly efficiently.

The apparent relationship between the ratio of mucosal to blood neutrophil counts and the presence or absence of fever in profoundly neutropenic patients is intriguing. Certainly, it is attractive to suppose that a direct measure of the delivery of neutrophils to tissues might supplement blood neutrophil counts in predicting the extent of a host defense defect and the risk of infection in such patients. However, our findings must be interpreted cautiously in this regard, since these studies were not specifically designed to address the question of infection risk. Moreover, the neutropenic patients who were studied represent a selected population. Nonetheless, our findings do suggest that the quantitative measurement of mucosal neutrophils deserves further consideration and study as a clinically useful tool in neutropenic patients.

ACKNOWLEDGMENT

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Modulation of In Vitro Growth of Murine Myeloid Leukemia by an Autologous Substance Immunochemically Cross-Reactive With Insulin and Antiinsulin Serum

By Z. Vuk-Pavlović, K. Pavelić, and S. Vuk-Pavlović

Murine myeloid leukemia secretes a substance immunochemically cross-reactive with insulin (SICRI) both in vivo and in serum-free media. High SICRI concentrations in peripheral blood of tumorous animals do not affect circulating glucose levels. In culture, DNA synthesis rate per leukemic cell is proportional to cell density and is reduced by antiinsulin serum. Culture medium conditioned by leukemia cells as well as SICRI affinity purified from this medium stimulate DNA synthesis in cultured leukemia cells. It appears that autocrine stimulation of murine myeloid leukemia can be mediated in part by an insulin-related growth factor.

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PROGRESSION of several human tumors is paralleled by elevated blood levels of substances immunochemically cross reactive with insulin (SICRI).[1-5] SICRI production in tumorous diabetics[2,3] and by cultured tumor cells[4] demonstrate that this substance is indeed of tumor origin. On the basis of circumstantial evidence, we speculated that SICRI might mediate tumor autostimulation by an endocrine and/or autocrine mechanism.[5-7]

Autostimulation in murine melanoma B16, a SICRI-secreting tumor, was recently quantitated by mathematical modeling.[8] However, this phenomenologic mathematical model cannot reveal the mechanistic involvement of SICRI in autostimulation. Here, in murine myeloid leukemia (MML),[9] we demonstrate in vitro the autocrine growth control partially mediated by SICRI.

MATERIALS AND METHODS

Male RF mice, eight to ten weeks old, were obtained either from Rugjer Bošković Institute, Zagreb, or from Jackson Laboratory, Bar Harbor, Me. They were kept five per cage and fed standard pelleted food and water ad libitum. Myeloic leukemia was maintained by IV inoculations of 1×10^6 cells obtained from spleens of diseased animals. Blood was drawn by axillar exsanguination. Cellularity of spleen was determined by weighing spleens by use of a Mettler microbalance and by counting suspended splenocytes in a hemocytometer.

Insulin and SICRI concentrations were determined by commercial double-antibody insulin-specific radioimmunoassay (RIA: Serono, Saluggia, Italy) or by kits assembled using commercial polyclonal antibodies (Cappel, Malvern, Pa) and A_{14}-monoiodotyrosine insulin (360 μCi/mg; Eli Lilly, Indianapolis, Ind). Therefore, SICRI concentrations are relative and expressed as insulin equivalents RIA artifacts were excluded by control experiments: (1) Incubation of ^{125}I-insulin with tumor extracts or concentrated conditioned tissue culture media (*v infra*) for 12 hours of room temperature (RIA conditions) neither significantly decreased the amount of phosphotungstic acid precipitable material nor changed the position of the radioactive peak in the eluates of these samples from a Protein Analysis I-125 HPLC column (Waters Associates, Milford, Mass). These experiments show the absence of falsely elevated SICRI concentrations due to ^{125}I-insulin degradation; (2) SICRI preparations failed to increase the amount of ^{125}I-insulin precipitated by 14% polyoxyethylene 6000 (POE) after 12 hours' incubation, demonstrating the absence of ^{125}I-insulin binding materials in these preparations; (3) Substitution of the second antibody by 18% POE and 1 mg/mL human IgG resulted in the same values as obtained by double-antibody RIA showing that SICRI containing samples do not interfere with binding of the second antibody. Growth hormone concentrations were measured by commercial double-antibody RIA (Serono, Saluggia, Italy). Glucose concentrations were measured by the glucose oxidase-peroxidase method (Sigma, St. Louis, Mo).

Spleens were weighted and homogenized in nine parts of phosphate-buffered saline, pH 7.5. The homogenate was centrifuged for 10 minutes at 10,000 g and SICRI in the supernate was measured by insulin-specific RIA.

Murine myeloid leukemia cultures were established from the leukemic spleen of a RF mouse. A cell suspension was prepared by passing tumor tissue through a nylon mesh and several times through a gauge 21 needle and cells were grown at 37 °C in serum-free transferrin-supplemented (5 mg/L; Sigma) Iscove's modified Dulbecco's medium (Sigma) in 5% CO_2. The medium was changed every second day. The first medium change was discarded and the subsequent ones were used as the source of SICRI.

Stimulation of DNA synthesis by growth factors was measured in cells grown in Costar (Cambridge, Mass) 96-well clusters by measuring [methyl-^{3}H]-thymidine (specific activity 2 Ci/mmol, 1 mCi/mL; Research Products International, Mt. Prospect, Ill) incorporation. The original [^{3}H]-thymidine solution was diluted tenfold with the cell culture medium and a dose of 0.2 μCi of [^{3}H]-thymidine in 2 μL was added to each well. Duration of incubation with [^{3}H]-thymidine is indicated in the legends to Figs 2 and 3 and Table 1. Cells were harvested using a Bellco cell harvester. Radioactivity of incorporated thymidine was measured by use of 3a70 scintillation cocktail (Research Products International, Mt. Prospect, Ill) and Packard Tri-Carb Liquid Scintillation Spectrometer.

SICRI preparations assayed for effects on [^{3}H-methyl]-thymidine incorporation by MML cells were obtained from serum-free tissue culture medium conditioned by tumor cells and concentrated by use of an Amicon UM2 membrane. This concentrated medium was dialyzed against PBS, pH 7 4, and used for cell culture experiments or was applied to a column of affinity purified guinea pig antiporcine insulin antibodies immobilized on Affi-Gel 10 (Bio Rad) according to manufacturer's instructions. The column was extensively washed with PBS and the bound material was eluted with 1.0 mol/L acetic acid. The eluate was neutralized immediately with 2 mol/L sodium hydroxide and dialyzed against PBS in Spectrophor dialysis bags (M.W. cut-off = 1000; Spectrum Medical Industries, Los Angeles, Calif).

The apparent relative molecular mass (M_r) of native SICRI was estimated by gel filtration of the concentrated conditioned medium on a 0.9 × 50 cm Sepharose B6 column. PBS containing 0.3 mg/mL bovine serum albumin at a flow rate of 10 mL · cm^{-2} · hr^{-1} was used

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as the mobile phase. Blue dextran, human IgG, human serum albumin, bovine ^{125}I-insulin, and potassium ferricyanide were used for column calibration. The fractions in PBS were assayed by RIA directly and those in 1 mol/L acetic acid only after removal of the acid by freeze drying and reconstitution with the RIA buffer.

Cell colonies were established in soft agar in 35 × 10 mm Petri dishes. The solidified bottom layer [2.5 mL of 0.5% agar in Dulbecco's modified Eagle's medium (DMEM) supplemented as indicated in Table 2] was overlaid with 0.85 mL of DMEM containing 5 × 10^4 MML cells and 0.3% agar. Monocomponent porcine insulin (Novo, Copenhagen, Denmark), SICRI, FCS, human transferrin (Sigma, St. Louis), and selenous acid (Sigma) were added at concentrations indicated in Table 2. Quadruplicate cultures were incubated at 37 °C in water-saturated air supplemented with 5% CO_2 for four days. Colonies were defined as clusters of more than 30 cells. Statistical analysis was performed by the Student's *t* test.

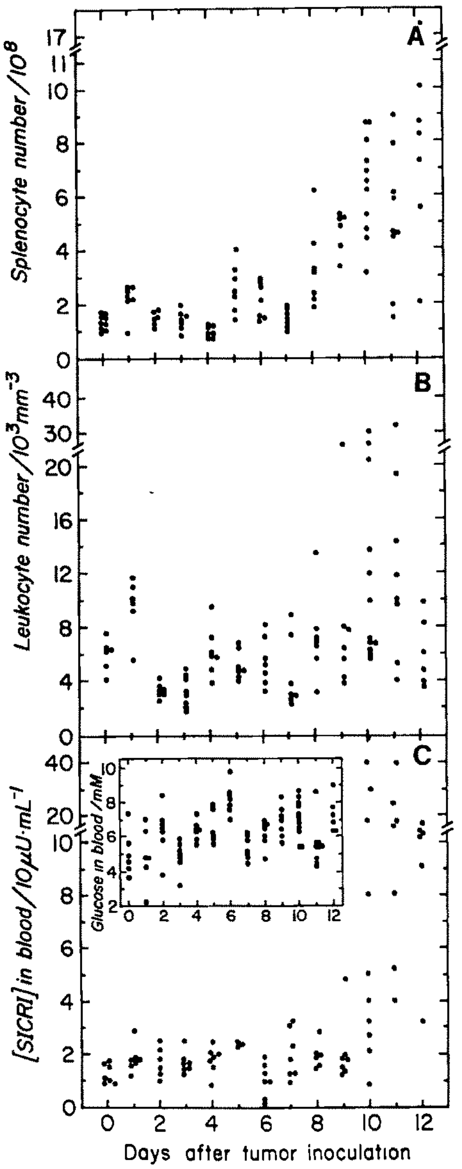

Fig 1. The time course of change of splenocyte number (A), peripheral blood leukocyte number (B), and of circulating SICRI (C) and glucose concentrations (inset) in mice IV inoculated with 1 × 10^6 myeloid leukemia cells on day 0.

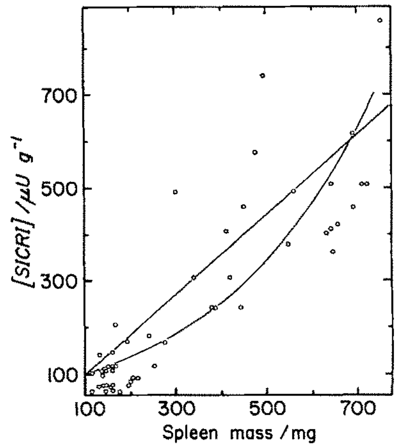

Fig 2. Intracellular SICRI concentration normalized per gram of wet spleens of RF mice suffering from myeloid leukemia as a function of spleen mass. Least-square fitting procedures yielded relationships [SICRI] = 850 M_s + 10.68 with R = 0.77 (straight line), and [SICRI] = 75.98 exp (3.01 M_s) with R = 0.76 (curved line), where SICRI concentrations are expressed in μU of insulin equivalents per gram of wet spleen and spleen mass, M_s, in grams.

RESULTS AND DISCUSSION

When one million murine myeloid leukemia cells were injected IV in RF mice, the animals died 12 days later.[10] Within the first seven days after inoculation, the spleen mass, number of peripheral leukocytes, and insulin levels in peripheral blood remained virtually normal. However, from the eighth day, these three parameters increased dramatically reaching maxima on the tenth day. Glucose levels did not change under the same circumstances (Fig 1). All these parameters, except glucose, decreased somewhat near the end of the survival period (days 11 and 12) when the animals were evidently cachectic. Growth hormone concentrations in peripheral blood were not affected by the disease.

We measured SICRI concentrations in homogenized leukemic spleens. In Fig 2, SICRI concentrations normalized per g of wet spleen tissue are plotted as a function of spleen

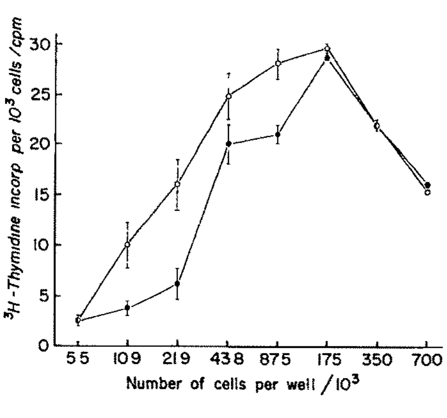

Fig 3. [^{3}H-methyl]-thymidine incorporation by murine myeloid leukemia cells in absence (○) and presence (●) of 1:1,000 diluted guinea pig antiporcine insulin antiserum as a function of cell density. Cells suspended in 0.2 mL of Iscove's modified Dulbecco's medium were placed in wells of Costar 96-well clusters. To each well 0.2 μCi of [methyl-^{3}H]-thymidine was added at the beginning of the 12-hour incubation.

mass. These data show that the increase of spleen mass, due to colonization by leukemic cells,[11] is paralleled by an increase of SICRI concentration in whole cell homogenate.

From the spleen of a tumorous mouse, we established the primary leukemia culture and propagated it in serum-free transferrin-supplemented (5 mg/L) Iscove's modified Dulbecco's medium (IMDM). These cultures were serum independent and they secreted SICRI into the medium. After three days of cultivation, media harvested from confluent cultures contained SICRI in concentrations up to 50 µU/mL.

The presence of autocrine growth promoting activity in media of MML cells was established by monitoring [methyl-^{3}H]-thymidine incorporation by MML cells as a function of cell density, both in presence and absence of antiinsulin serum for 12 hours (Fig 3). The normalized DNA synthesis rate, as measured by thymidine incorporation per cell, depended strongly on cell density: at densities up to 1×10^5 cells/well, it was proportional to the number of cells, while at higher cell densities, it was inversely proportional. The addition of antiinsulin serum (1:1,000 dilution) to these cultures interfered with DNA synthesis throughout the range of synthesis proportional to cell density (Fig 3). Indeed, up to the density of $\sim 2 \times 10^4$ cells/well, this serum almost completely inhibited DNA synthesis. To evaluate whether these inhibitory effects are due to toxicity of antiinsulin serum, we performed experiments with longer incubation with the antiserum (Table 1). Thirty thousand cells were incubated for 24 (rather than 12) hours with different dilutions of antiserum. Then [^{3}H]-thymidine was added for three more hours. Although different experimental conditions preclude other comparisons between data in Fig 3 and Table 1, it is obvious that the 1:800 serum dilution was not cytotoxic (Table 1); therefore, data in Fig 3 reflect neutralization of SICRI by antiinsulin serum. Moreover, data in Table 1 showed that this effect of serum depended on serum concentration and that at 1:200 dilution it could reduce the DNA synthesis by one half (Table 1). It appears, therefore, that stimulation of the DNA synthesis rate is due to a higher concentration of autocrine growth factors at higher cell densities. The inhibitory effects of antiinsulin serum not only support this notion, but demonstrate the existence of an insulin-related growth factor.

By use of concentrated and dialyzed IMDM conditioned by MML cells, we were able to modulate MML growth in vitro (Fig 4). The addition of SICRI up to 64 µU/mL stimulated DNA synthesis by MML cells; at higher concentrations DNA synthesis was reduced. Qualitatively identical data were obtained by use of SICRI, affinity purified from MML-conditioned medium on an antiinsulin-agarose column (inset to Fig 4). The extent of stimulation of DNA synthesis by this preparation is smaller than by unfractioned conditioned medium, yet the maximum stimulation is obtained at the same SICRI concentration (64 µU/mL). Moreover, like the cells treated with crude conditioned medium, the cells treated with affinity-purified SICRI showed a diminished incorporation of thymidine at SICRI concentrations above 64 µU/mL. This type of biphasic concentration-response relationship is similar to that seen with other growth-promoting substances.[12]

Table 1. Effect of Antiinsulin Antiserum on Growth of Cultured MML Cells

Dilution	[Methyl-^{3}H]-thymidine Incorporation/cpm Normal Guinea Pig Serum	 Guinea Pig Antiinsulin Antiserum	Inhibition/%
1/800	2035 ± 244	2077 ± 192	0
1/400	2375 ± 232	1839 ± 122	23
1/200	2190 ± 255	1158 ± 94	47

In each well 30,000 cells in 0.2 mL IMDM were incubated with sera for 24 hours when 0.2 µCi [methyl-^{3}H]-thymidine in 2 µL was added. Cells were harvested three hours later.

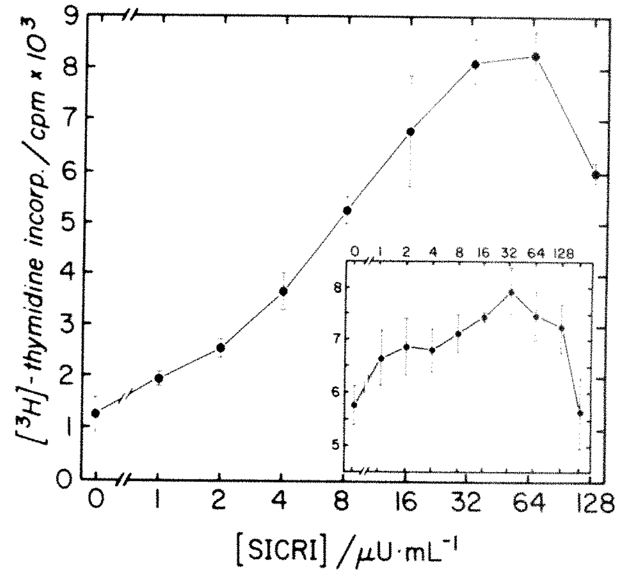

Fig 4. [^{3}H-methyl]-thymidine incorporation by 3×10^4 murine myeloid leukemia cells as a function of SICRI concentration. SICRI was added either in the form of concentrated and dialyzed (through a MW cut-off = 1000 membrane) medium conditioned by myeloid leukemia cells or as an affinity-purified preparation from the same conditioned medium (inset). Cells were incubated with SICRI for 12 hours when [methyl-^{3}H]-thymidine was added for 5 more hours.

We compared the effects of SICRI and insulin on colony formation by MML cells in soft agar (Table 2 and Fig 5). Insulin (0.25 µU/mL) did not stimulate colony formation, but the equivalent concentrations of SICRI exerted significant stimulation ($P < 0.01$). This finding is in full agreement with results of analogous experiments performed with melanoma B16 cells[16] and L1210 cells (K. Pavelić, unpublished results).

Table 2. Effects of Insulin and Affinity-Purified SICRI on Colony-Forming Ability of MML Cells in Soft Agar

Treatment	Number of Colonies
DMEM	98 ± 16
DMEM + 10% FCS	479 ± 70
Insulin (0.25 µU/mL)	128 ± 26
SICRI (0.25 µU/mL)	237 ± 46
SICRI (0.50 µU/mL)	336 ± 58

Human transferrin (5 mg/L) and selenous acid (25 µg/L) were added to media without FCS.

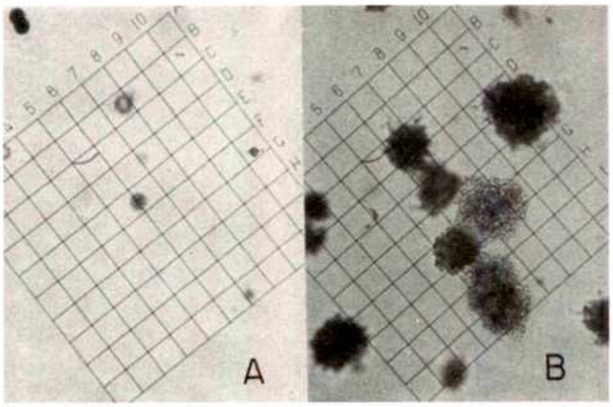

Fig 5. **Colony formation by MML cells in soft agar in the presence of (A) 0.25 μU/mL insulin and (B) 0.25 μU/mL SICRI, in serum-free Dulbecco's modified Eagle's medium supplemented with human transferrin (5 mg/L) and selenous acid (25 μg/L). (Original magnification × 400, current magnification × 220).**

By control experiments, we excluded possibilities of falsely positive SICRI measurements which could have arisen by proteolysis of ^{125}I-insulin and by the presence of other mitogens in SICRI preparations. Parallelity of SICRI concentration and mitogenic potency in affinity-purified SICRI samples is in line with this notion.

The antiinsulin sera employed in our experiments were obtained by immunization of guinea pigs with pure porcine insulin. Antibodies immobilized on agarose gels were previously affinity-purified themselves on an insulin-agarose column. In view of the apparent lack of interference of proinsulin and insulin in the RIA procedures for insulinlike growth factors I and II (IGF I and II),[13] identity of these growth factors and SICRI is not very probable. However, IGF I stimulated growth of cultivated MML cells in a dose-dependent manner up to the concentration of 25 ng/mL (S. Vuk-Pavlovič, unpublished data).

From gel-permeation chromatography data obtained with a Sepharose 6B column under nondenaturing conditions (Fig 6), we calculated the M_r value of native SICRI to be 120,000. Similar values were reported for SICRI in sera of non-Hodgkin's lymphoma patients[3] and in serum-free media cultivated by murine melanoma B16.[16] However, we were not able to assess the M_r value of SICRI in 1 mol/L acetic acid due to our failure to detect any insulinlike material after freeze-drying and reconstitution of chromatographic fractions.

We demonstrate that murine myeloid leukemia in RF mice is accompanied by an increase of SICRI in the whole cell homogenate and circulation. This increase does not affect glucose levels in circulation. Cells of this tumor are serum-independent in culture and secrete SICRI in the medium. Moreover, we conclude that SICRI stimulates growth of SICRI-secreting cells because antiinsulin serum interferes with DNA synthesis dependent on cell density and because affinity-purified SICRI promotes cell growth. Thus, in MML cells, all the elements necessary for establishment and maintenance of autocrine stimulation are present. However, the different extent of stimulation of DNA synthesis by purified SICRI and conditioned IMDM reveals that MML cells produce other growth factors and are stimulated by them.

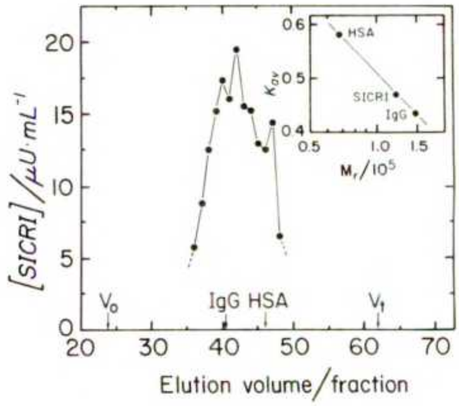

Fig 6. **Molecular sieving chromatography of SICRI on a Separose 6B 0.9 × 50 cm column developed with phosphate-buffered saline containing 0.3 mg/mL bovine serum albumin at a flow rate of 10 mL · cm^{-2} · hr^{-1}. The column was calibrated with blue dextran (V_o, void volume), human immunoglobin G (IgG), human serum albumin (HSA), and potassium ferricyanide (V_t, total volume). Inset: Plot of K_{av} [$=(V_e - V_o)/(V_t - V_o)$ where V_e denotes the elution volume], v log apparent relative molecular mass (M_r) of HSA, SICRI, and IgG.**

Our results show that tumors can produce and secrete substances related to insulin and that these products can serve as tumor growth regulators. These findings suggest an analogy with the expression and role of insulin and related factors in prenatal life,[14] inasmuch as similarities between endocrine aspects of cancer and embryology exist. Also, origins and effects of tumor-produced insulin-related substances seem to mirror other ectopically produced growth factors in tumors.[15]

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The Chemokinetic Response of Human Neutrophils

By Thomas H. Howard

A computer-assisted single cell assay that allows quantification of the locomotive behavior of individual cells and a flow-through system that allows study of response of individual cells to stimulation were utilized to study the chemokinetic response of neutrophils. The range of basal mean rate of locomotion (mROL) and chemokinetic response to 10^{-9} mol/L formylmethionyl leucyl phenylalanine (fMLP) was determined for neutrophils of eight normal adults. The basal mROL was 8.2 ± 1.5 um/min and 6.2 ± 1.0 um/min; the rate after 10^{-9} mol/L fMLP was 12.1 ± 2.1 and 9.5 ± 1.8 um/min in 2.0 g% and 0.05 g% HSA, respectively. The mean increase in ROL for neutrophils was 50%. Assay with the flow-through system shows that the chemokinetic response—increase in mROL of a population of neutrophils in response to 10^{-9} mol/L—is due to an increase in ROL when cells are actively moving and not due to a decrease in the amount of time the cell spends inactive. Studies of individual cells within the populations show that chemokinetic response to 10^{-9} mol/L fMLP is highly variable. The majority of cells (77%) respond with an increase in ROL; the minority (23%) are nonresponders that characteristically move at ROL $\geq$ 14 um/min prior to stimulation and do not change ROL or exhibit a net decline in ROL in response to 10^{-9} mol/L fMLP. The dose response of a population of neutrophils and of individual neutrophils to serial addition of 10^{-10} to 10^{-6} mol/L fMLP shows that the fMLP dose dependence for maximal chemokinetic response is highly variable among individual cells. Seventeen percent of cells do not respond to any fMLP concentration; 25% of neutrophils exhibit maximal response to 10^{-10} mol/L fMLP, while 50% and 25% of cells showed peak chemokinetic response to 10^{-9} mol/L and $\geq 10^{-8}$ mol/L fMLP, respectively. These studies document the variability in the locomotive responses of peripheral blood neutrophils. Understanding the causes of variability in the chemokinetic responsiveness of individual neutrophils may improve our understanding of how the cellular inflammatory response in man can be modulated.

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THE original descriptions of neutrophil locomotion by Sabin,[1] McCutcheon,[2] and Debruyn[3] utilized methods that quantified the locomotive behavior of individual cells. After the early 1950s the Boyden chamber[4] and the chemotactic assay under agarose[5] replaced these single cell methods. The Boyden chamber and agarose assay are less tedious and more readily controlled than single cell methods; however, these new techniques yield less information about the locomotive behavior of neutrophils than do single cell techniques. For example, without major modifications[6] the Boyden chamber and the assay under agarose cannot (A) measure the actual rate of movement of cells; (B) recognize subpopulations of cells that differ in locomotive behavior; or (C) clearly segregate the orientational response and the rate response (chemokinesis)[7] of neutrophils to a chemotactic factor.

These limitations of the Boyden chamber and the agarose assay prompted a return to single cell assays[7–14] to investigate the locomotive behavior of neutrophils. Single cell studies have yielded important information on the range of rates of locomotion for neutrophils,[9,11,13] quantitative description of the irregularity of locomotion,[9,10,12,14] information on the turning behavior of neutrophils,[9,11,15] and allowed analysis of the ability of neutrophils to orient in gradients of chemotactic factor.[7] However, a careful study of the chemokinetic response of neutrophils and the variability in the chemokinetic responsiveness of individual neutrophils has not been reported. For this reason we developed a single cell method that is reproducible and readily controlled.[8] We paired the single cell assay with a flow-through system that allows quantification of the locomotive behavior of the same cells before and after addition of a chemotactic factor and we applied the assay to quantitatively describe the chemokinetic response of neutrophils to formylmethionyl leucyl phenylalanine (fMLP).

MATERIALS AND METHODS

Neutrophils were prepared by the method of Boyum[16] on Ficoll-Hypaque gradients. Resultant preparations were >98% granulocytes with 95% to 96% neutrophils and 4% to 5% eosinophils. The cells were suspended in HEPES/Hanks Balanced Salt Solution (H/HBSSG) (25 mmol/L HEPES, 50 mmol/L Phosphate, 150 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl, 1 mmol/L $CaCl^2$, pH 7.15) with 150 mg% glucose and delipidated human serum albumin (SIGMA) as noted. The fMLP (SIGMA) was dissolved in DMSO Equipment utilized in the single cell assay includes the Dvorak-Stotler tissue culture chamber (Nicholson Precision, Bethesda, Md); infrared incubator (Opti-Quip, Highland Mills, NY); telethermometer and banjo probe (YSI, Yellow Springs, Ohio); Leitz Dialux 20 Microscope with 16X bright field objective; Ikegami vidicon videocamera, FORMA time-date generator; Panasonic NV-8030 time lapse videotape recorder; Ikegami videomonitor; and GP60 Sonic digitizer (Science Accessories Co, Norwalk, Conn). The remainder of the details of the assay are given in the text.

Description of single cell assay. Neutrophils at 4 °C are injected into a tissue culture chamber that is servo-linked to an infrared incubator via a heat-sensitive gold probe tapped into the chamber frame The visualization chamber is formed by two coverslips that are 13 mm apart. The temperature of the lower coverslip—the coverslip to which cells are attached—was monitored with a banjo probe sealed to that coverslip and linked to a telethermometer

Cells were injected into the chamber, the chamber was placed on a microscope stage, warmed to 37 °C (coverslip temperature), and allowed to equilibrate at 37 °C for 10 minutes. Using a videocamera, the cells were visualized as 3 to 4 mm black dots on a videomonitor

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and were time-lapse videorecorded at 18 times real time for the specified periods of observation.

Following videorecording, the paths taken by the cells were tracked onto plastic transparencies as previously described.[9] The initial position of each cell was marked; the videorecording was advanced a specified period of time, the position of each cell was again marked; and the process was repeated until the desired period of observation had elapsed. In each case, the elapsed time between observations and total time of observation is indicated in Figure legends.

The transparency was placed on the digitizing board of a sonic digitizer/microprocessor and the Cartesian coordinates of each cell at each time of observation were entered into a computer file as a series of six digits following a cell identifier. The working file was entered into an interactive program that allows scaling and feature selection. The program computes the rate of locomotion (ROL) of each cell (ROL = distance travelled/total time observed); the active rate of locomotion (aROL = distance travelled/total time minus time inactive); and the number of 0.5 minute inactive intervals/cell/5 min of observation (0.5 min II/cell/5 min). The terms mean ROL (mROL) and mean active ROL (maROL) are designations used to describe the average locomotive parameter for a population of cells. The terms ROL and aROL are used for individual cells.

Our controls for reproducibility of the single cell assay show that (1) the mROL for a population of cells varies less than 10% during 20 minutes of observation; (2) the mROL of a population of cells from one individual varies <10% from the mean on each of five different days; (3) the mROL of a population is not affected by in vitro age of cells over the range of 3 to 6 hours; (4) the mROL is not affected by cell densities ranging from 45 cells/field to 250 cells/field; (5) the mROL is affected by human serum albumin (HSA) concentration in the H/HBSSG buffer.[9] The HSA (g%) is given in each figure legend.

The flow-through system. For experiments done with the flow-through system, cells at 4 °C are injected into the culture chamber, which is mounted in a plexiglass chamber containing immersion oil. The cells, chamber, and oil are warmed to 37 °C and stabilized for 10 minutes. Cells are videorecorded as noted above during a control period (≥5 minutes), then, during an infusion period, the specified concentration of fMLP or DMSO in H/HBSSG is pumped into the chamber with a Harvard pump (rate 0.220 ml/min) for five minutes; the response period follows fMLP addition. Videorecording continues during the infusion period and during the response period to allow tracking and analysis of the same cell(s) during both the control period and the response period The temperature is maintained at 37 °C during the infusion. The duration of the infusion period and rate of flow is adequate to raise the fMLP concentration to 97% of the influent concentration as documented by infusion of ^{3}[H] fMLP and measurement of cpm from ^{3}[H] fMLP in the effluent. Since flow under these conditions is turbulent (indicated by elution curve and chamber description[11]), no gradient of fMLP exists at any time. This was documented by determining the direction of cell locomotion in the plane of the cover slip The direction of cell locomotion is random; hence, we are studying exclusively the effect of fMLP on rate of locomotion (chemokinesis).

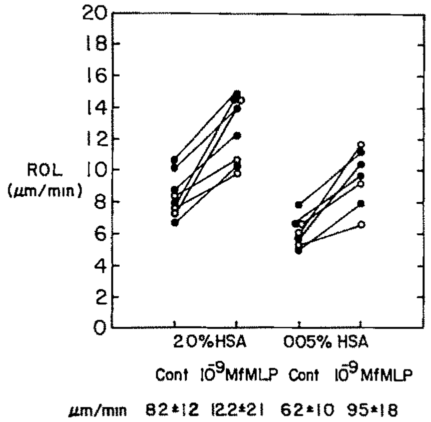

Fig 1. The basal mROL and fMLP-induced chemokinetic response of neutrophils from normal adults. Each point is the mROL of 75 to 100 neutrophils from one subject either male (●) or female (○). The neutrophils were studied in their basal state (cont) or the stimulated state (10^{-9} mol/L fMLP) in the specified concentration of human serum albumin (HSA). Basal and stimulated values for the neutrophils of each subject are connected with a line.

For the serial fMLP dose response, cells are observed in a control period with DMSO (0.1% v/v) present, a five-minute infusion period to 10^{-10} mol/L fMLP follows; then a 5-minute 10^{-10} mol/L fMLP response period; then the infusion of increasing one log steps in fMLP concentrations (10^{-9} – 10^{-6} mol/L) alternated with five-minute response periods.

RESULTS

The chemokinetic response of neutrophils from normal adults. The single cell assay was utilized to determine the basal mROL of normal neutrophils and the chemokinetic effect of fMLP on normal neutrophils. Studies were done in the presence of 0.05 g% and 2.0 g% of HSA. As shown in Fig 1, the basal mROL of neutrophils from five normal males and three normal females is 8.2 ± 1.5 and 6.2 ± 1.0 um/min in 2.0 g% and 0.05 g% HSA, respectively. These values are the mean ± one SD.

Neutrophils from the same normal individuals were also exposed to a uniform concentration of 10^{-9} mol/L fMLP and the chemokinetic response of the neutrophils was determined. As shown in Fig 1, the mROL of neutrophils exposed to 10^{-9} mol/L fMLP is 12.1 ± 2.1 and 9.5 ± 1.8 um/min in the presence of 2.0 g% and 0.05 g% HSA, respectively. The mean percent increase in mROL of neutrophils exposed to 10^{-9} mol/L fMLP was 50% above basal values (range 30% to 70%).

Analysis of the locomotive behavior of neutrophil populations during the chemokinetic response. During four separate trials, 40 to 60 neutrophils were tracked continuously during a five-minute control period, a five-minute infusion period when the concentration of fMLP was raised to 10^{-9} mol/L, and a 5- to 20-minute chemokinetic response period. Quantitative analysis of the continuous tracks yields the mean rate of locomotion (mROL), the mean *active* rate of locomotion (maROL), and the number of 0.5-minute inactive intervals/cell/5 min (#0.5 min II/cell/5 min) for the entire population of cells. Results are shown in Fig 2. The mROL is 9.9 um ± 2.0 um/min during the control period and 17 ± 1.8 um/min during the first five minutes of the response period. This represents a 70% increase in the mROL of the population of neutrophils. The mROL for the population remains elevated for at least 15 minutes after stimulation (15.8 ± 1.8 um/min for the second five minutes, and 16.2 ± 2.2 um/min for the third five minutes of the response period).

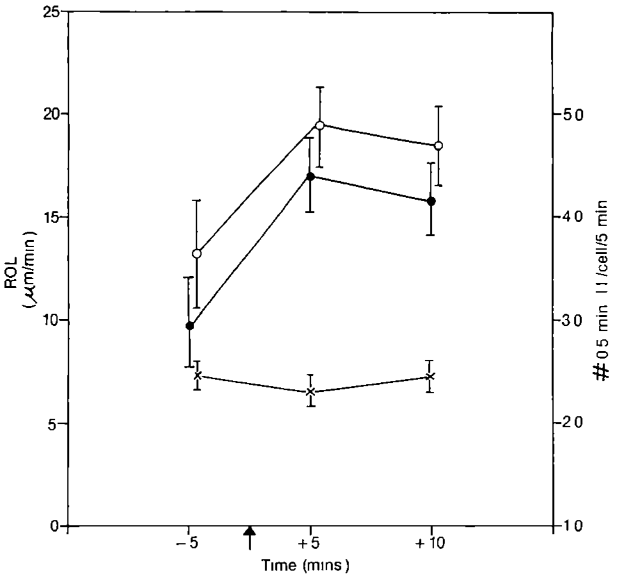

Fig 2. Chemokinetic response of neutrophils to 10^{-9} mol/L fMLP: Locomotive parameters (●—●) mROL (mean rate of locomotion); (○—○) maROL (mean active rate of locomotion) and (x—x) number of 0.5 minute inactive intervals/cell/5 min (#0.5 min I.I./cell/5 min) were measured before (−) and after (+) addition of 10^{-9} mol/L fMLP (↑). Presented is mean ± SD from four trials with 40 to 60 cells studied in each trial. Studies were done in 0.5 g% HSA, cells tracked at 0.5-minute intervals.

In our assay[9] and assays described by others,[10,14] neutrophils are not continuously in motion. Their course is interrupted by periods of inactivity (inactive intervals). Therefore, any increase in the mROL of cells could reflect either a decrease in inactive intervals or an increase in maROL, ie, the rate of locomotion during periods when cells are actively moving. To determine whether the chemokinetic response reflects an increase in maROL, a decrease in inactive intervals, or a combination of both, we determined the maROL and the number of 0.5-minute inactive intervals/cell/5 min before and after fMLP.

An increase in the maROL for the population accompanies the increase in mROL. The maROL for the population is 13.2 ± 2.5 um/min during the control period and 19.2 ± 1.8 um/min during the first five minutes of the response period. The maROL remains elevated for at least 15 minutes and the differential between mROL and maROL is constant (2.4 to 2.6 um/min) during that time. No consistent change in the number of 0.5 min II/5 min was observed (2.0 ± 0.6 before and 1.8 ± 0.6, 2.1 ± 0.6 after fMLP). These results show that the chemokinetic response of a population of neutrophils is due to an increase in the maROL of cells (ie, when cells move they move more rapidly), and is not due to a decrease in the amount of time spent inactive.

The chemokinetic response of individual cells within a population. While mean changes in locomotive parameters (mROL, maROL, #0.5 min II/cell/5 min) for a population of neutrophils clearly describe a chemokinetic response for the population as a whole, the response of individual cells within the population varies widely. This variability is reflected in the plots of the ROL of 25 randomly selected cells as a function of 2.5-minute intervals before and after

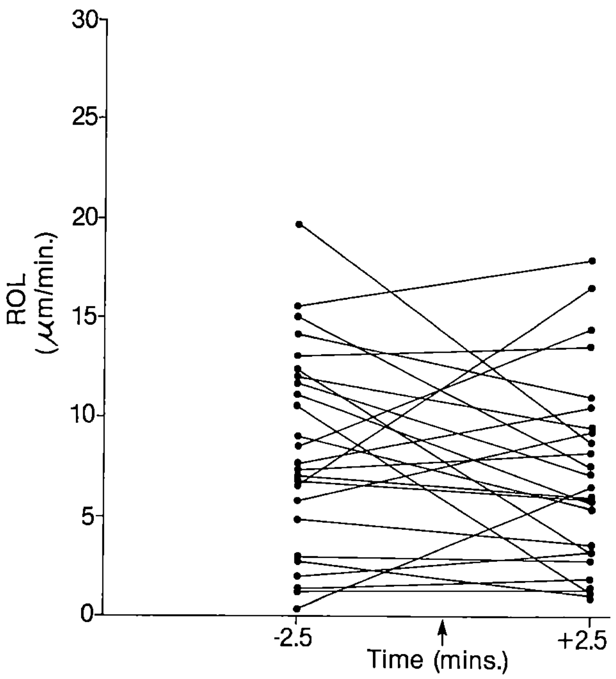

Fig 3. Rate of locomotion (ROL) of individual neutrophils before and after exposure to DMSO: The ROL (um/min) of 25 randomly selected cells before and after exposure to DMSO (0.1% vol/vol), the fMLP solvent is shown. At arrow (↑) DMSO was pumped into the chamber during 5 minutes. X-axis is time before (−) and time after (+) DMSO addition. Cells were studied in 0.5 g% HSA and tracked at 0.5-minute intervals.

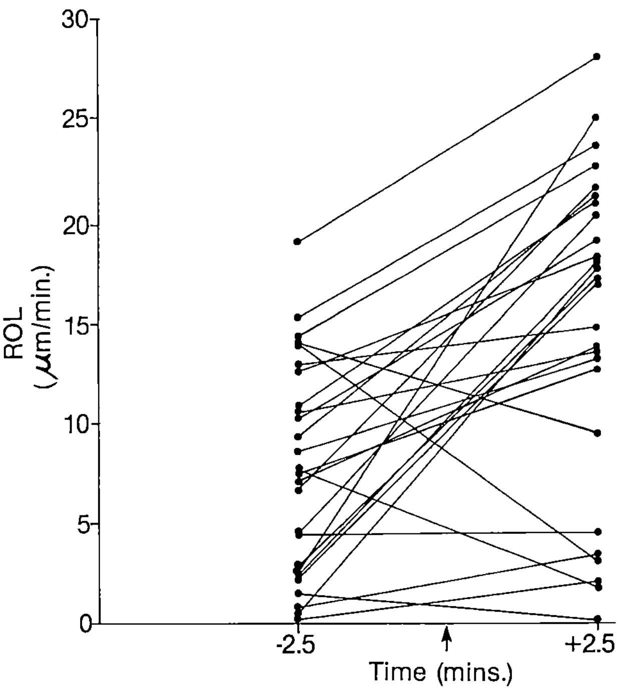

Fig 4. Rate of locomotion (ROL) of individual neutrophils before and after exposure to 10^{-9} mol/L fMLP: The ROL (um/min) of 25 randomly selected cells before and after exposure to 10^{-9} mol/L fMLP is shown. At arrow (↑) 10^{-9} mol/L fMLP was pumped into the chamber during 5 minutes. X-axis is time before (−) and time after (+) fMLP addition. Cells were studied in 0.5 g% HSA and were tracked at 0.5-minute intervals.

exposure to 10^{-9} mol/L fMLP or DMSO in the flow-through system (see Figs 3 and 4). The ROL for individual cells within a population of control- or fMLP-treated cells varies remarkably. For individual fMLP-treated cells it is clear that the trend is toward an increase in ROL; however, some cells exhibit no change in ROL or a definite decrease in ROL after fMLP is introduced Analysis of cells tracked for 10 minutes after fMLP addition show that the most remarkable change in ROL of individual fMLP-stimulated cells is obvious in the 2.5 minutes immediately following completion of fMLP addition and that the cells that respond to fMLP addition with a decrease in ROL never regain their original ROL even after 10 minutes exposure to fMLP; this is not true of control cells. Even if the ROL of an individual control cell decreases, the original or a greater ROL is subsequently observed.

To better define the response of cells to fMLP addition, we plotted the distributions of change in ROL (ΔROL) between the 2.5 minutes prior to and 2.5 minutes following control solvent or fMLP addition. The distributions are plotted both as histograms (number of cells versus ΔROL; Fig 5A) and as cumulative percentage of cells exhibiting equal to or less than a specific ΔROL (ΔROL = ROL 2.5 min following addition minus ROL 2.5 min prior to addition; Fig 5B). The distribution of cells exposed to control solvent is a normal distribution, ie, ~54% responded with no change or a decrease in ROL; 46% responded with an increase in ROL. This is an expected result if the changes in ΔROL are random following control solvent addition. In contrast, the distribution of ΔROL for cells exposed to 10^{-9} mol/L fMLP is markedly skewed toward higher values with 78% of cells exhibiting a gain, ΔROL >0, and 50% responding with a ΔROL of $\geq +5$ um/min. If we use a ΔROL of ≤ 0 um/min to define cells that do not respond to fMLP, then 21% of the 145 cells studied show no response to fMLP stimulation. These cells are nonresponders.

To characterize the locomotive behavior of nonresponders and to compare them to responders, we determined the mean ΔROL of cells as a function of the ROL of cells in the 2.5 minutes prior to 10^{-9} mol/L fMLP addition. As shown in Fig 6, the mean ΔROL for cells that moved <14 um/min prior to fMLP addition was $\geq +5$ um/min; in contrast, the mean ΔROL for cells that moved ≥ 14 um/min prior to fMLP addition was −0.5 um/min. The difference in the means is statistically significant ($P = 0.01$) by t test. This result indicates that the majority of cells with greatest ROL prior to fMLP addition do not exhibit a chemokinetic response to fMLP. In our experimental system it is unusual for cells to exhibit ROLs > 20 um/min before fMLP addition or after control solvent addition. However, after fMLP addition cells commonly attain ROLs > 22 um/min and as high as 33 um/min. Therefore, it is unlikely that cells moving at ≥ 14

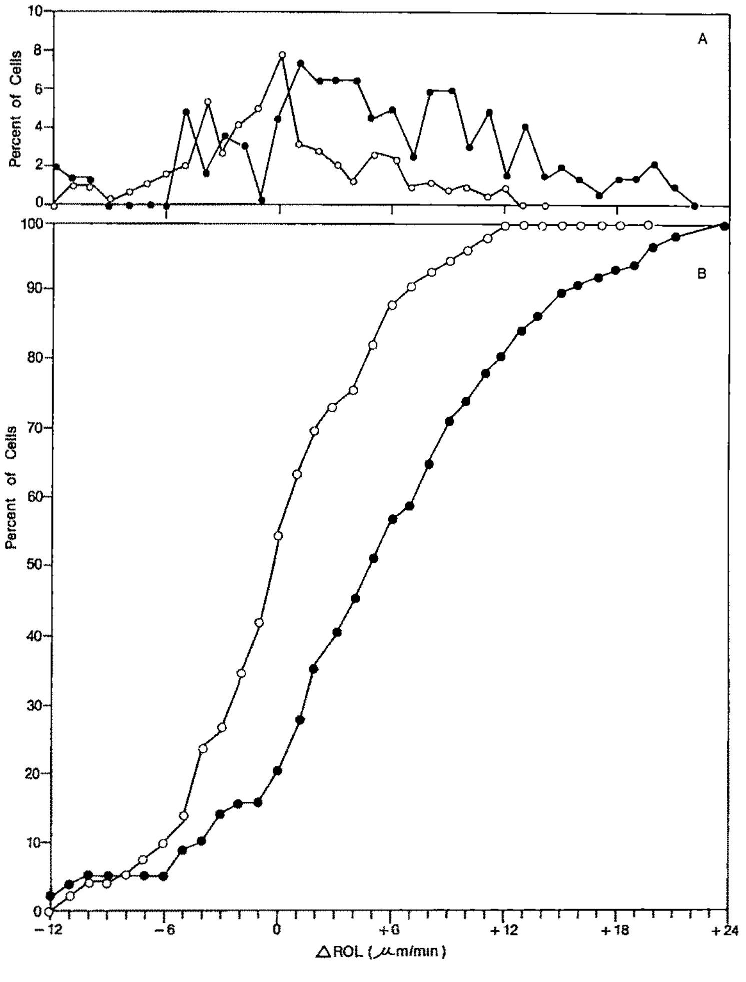

Fig 5. Distribution of ROL for 10^{-9} mol/L fMLP-stimulated neutrophils: (A) Percentage of cells exhibiting a specific change in ROL plotted as a histogram. (B) Same data presented as cumulative percentage of cells exhibiting a specific change in ROL. ΔROL is the difference in ROL of a cell 2.5 minutes prior to and 2.5 minutes following addition of 10^{-8} mol/L fMLP (●—●) or the control solvent DMSO (0.1% v/v) (○—○). The distributions are plotted from 75 cells observed with DMSO and 145 cells with 10^{-8} mol/L fMLP. Cells were studied in 0.5% HSA, tracked at 0.5-minute intervals.

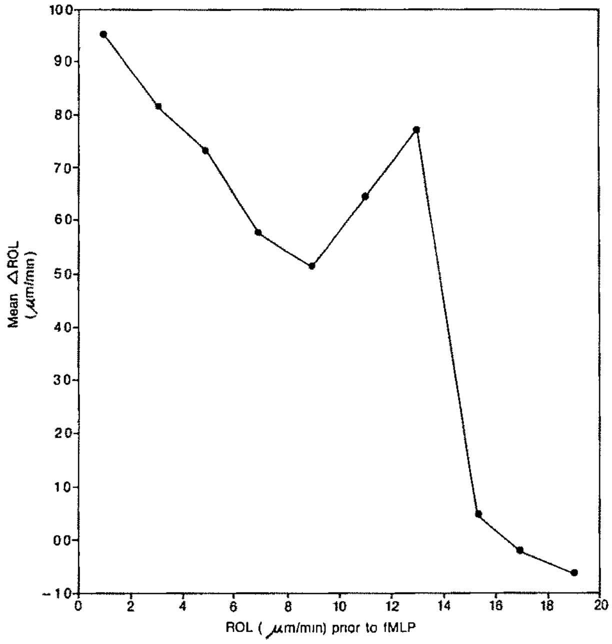

Fig 6. Relationship of mean Δ ROL of 10^{-9} mol/L fMLP-stimulated neutrophils to ROL before stimulation: The mean ΔROL was determined from Σ ΔROL/number of cells with indicated ROL prior to 10^{-9} mol/L fMLP. Y-axis is mean ΔROL; x-axis is ROL in 2.5-minute period prior to fMLP addition. Data reflects analysis of 145 cells.

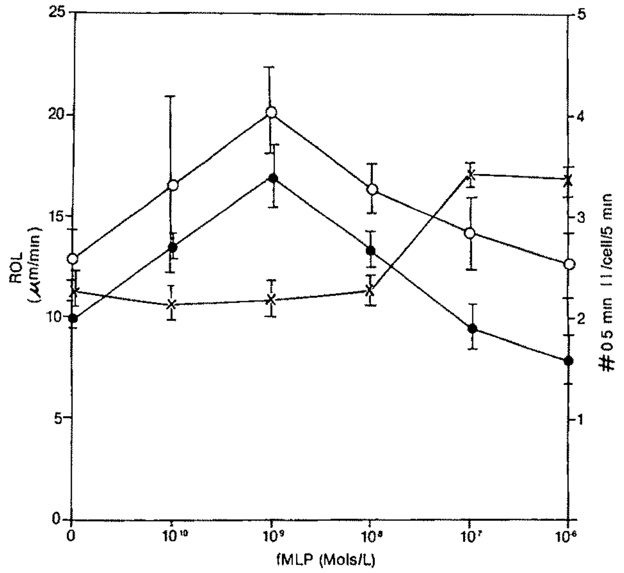

Fig 7. Dose dependence of chemokinetic response to serial addition of fMLP (10^{-10} to 10^{-6} mol/L): Neutrophils were exposed to serial increases in fMLP with 5-minute periods of observation. The (●–●) mROL (mean rate of locomotion), (O—O) maROL (mean active rate of locomotion), and (x—x) number of 0.5 minute inactive intervals/cell/5 min (#0.5 min I.I./cell/5 min) were determined. Cells were studied in 0.5 g% HSA and were tracked at 0.5-minute intervals during the response period. Shown are mean values ± SD for the entire population.

um/min prior to fMLP addition fail to exhibit a chemokinetic response because they are moving at their maximum possible ROL.

Response of locomotive parameters to serial addition of fMLP (10^{-10} to 10^{-6} mol/L). We previously published the dose response for mROL of populations of neutrophils exposed to increasing concentrations of fMLP (10^{-10} to 10^{-6} mol/L).[18] In the previous report, cells were exposed to a single dose of fMLP (10^{-10} mol/L, or 10^{-9} mol/L, or . . . 10^{-6} mol/L) and the mROL of the population was determined. We found that the maximum chemokinetic response occurred at 10^{-8} mol/L fMLP and at concentrations $>10^{-8}$ mol/L the mROL declined to control levels.[18] In the present studies, we used the flow-through system to characterize the dose response of chemokinesis for a population of neutrophils that are serially exposed to incremental increases in fMLP (10^{-10} mol/L and 10^{-9} mol/L and . . . 10^{-6} mol/L). This protocol of serial additions more closely mimics the chemokinesis that would be observed for neutrophils moving in a gradient of chemotactic factor; however, in the experiments presented here no gradient exists.

The maximal mROL for the entire population (Fig 7), ie, the maximal chemokinetic response, occurs with 10^{-9} mol/L fMLP and then mROL declines to control levels at fMLP concentrations $\geq 10^{-8}$ mol/L fMLP. To determine whether the decrease in mROL at $>10^{-8}$ mol/L fMLP was due to an increase in time spent inactive or a decrease in ROL when cells are actively moving, we determined the number of inactive intervals and the maROL of cells as a function of fMLP dose. With serial exposures to fMLP $\leq 10^{-8}$ mol/L the mean number of 0.5 min II/cell/5 min remains constant, the maROL is increased and the difference in maROL and mROL (maROL—mROL) remains constant. These results verify the early studies with 10^{-9} mol/L fMLP alone, ie, the neutrophils increase their mROL because they move faster when they move and not because they spend less time inactive. At fMLP concentrations $>10^{-8}$ mol/L fMLP, the mean number of inactive intervals increases to 3.3/cell/5.0 min, the maROL (13.0 um/min for 10^{-6} mol/L fMLP; 13.0 um/min for control) remains elevated while the mROL drops slightly below control levels (9.0 um/min for 10^{-6} mol/L fMLP; 10.0 um/min control) and the difference between maROL and mROL increases (3.7 to 4.6 um/min at $\geq 10^{-7}$ mol/L; 2.6 to 3.1 um/min at $<10^{-8}$ mol/L). These results indicate that at $>10^{-8}$ mol/L fMLP, the mROL of cells decreases both because the cells spend more time inactive and because they move at slower rates when active. However, the major change is an increase in time inactive.

Figure 8 presents the distribution curves for the maximal ROL observed for individual cells as a function of fMLP concentration. Seventeen percent of cells do not respond with an increase in ROL regardless of fMLP concentration, ie, these cells are nonresponders regardless of fMLP dose. The data show that for cells that exhibit a chemokinetic response to fMLP, the majority of the cells exhibit a maximal chemokinetic response at 10^{-9} mol/L fMLP, and the remaining cells are normally distributed around this value, ie, 25% with maximal ROL at 10^{-10} mol/L fMLP and 25% at $\geq 10^{-8}$ mol/L fMLP.

DISCUSSION

We utilized methods that allow the study of individual neutrophils and populations of neutrophils in order to quantitatively describe the fMLP-induced chemokinetic response

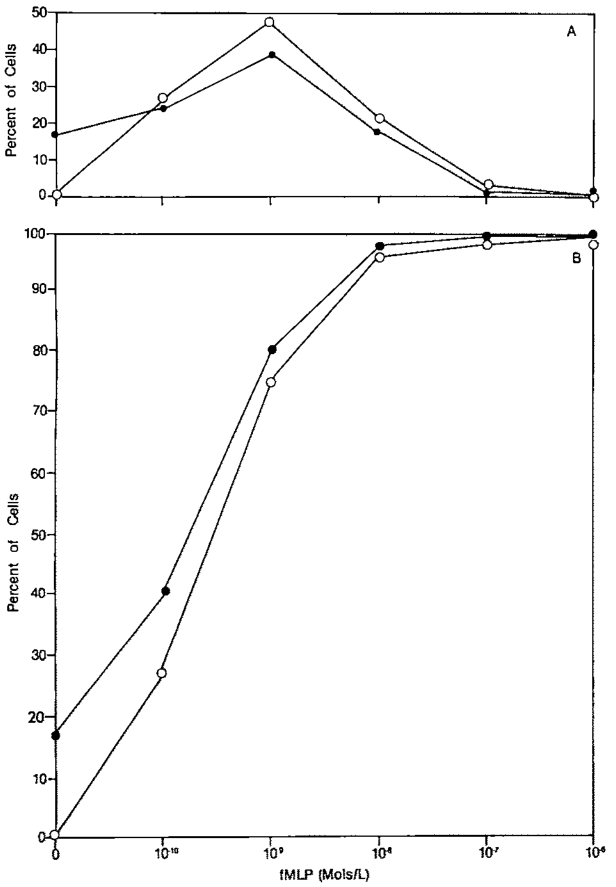

Fig 8. Dose dependency of maximal chemokinetic response of neutrophils: (A) Percentage of cells exhibiting maximal ROL as a function of fMLP dose. (B) Same data presented as cumulative percentage of cells exhibiting maximal ROL as a function of fMLP dose. Data for all cells (●——●) and data for responders (○——○) only are shown. ROL was determined for cells in 0.5 g% HSA, tracked at 0.5-minute intervals for 5 minutes.

of these cells. We investigated the variability in chemokinetic response among adult donors and used a flow-through system (1) to analyze the changes in active ROL and inactive intervals during the chemokinetic response of a population of cells; (2) to describe the variability in chemokinetic response among individual neutrophils in a population; and (3) to analyze the fMLP dose dependency of the chemokinetic response for a population of cells exposed to serial additions of fMLP. Our studies show that chemokinetic responsiveness of ficoll-hypaque purified neutrophils from normal adults is surprisingly constant, and 10^{-9} mol/L fMLP causes a 50% increase in the ROL of neutrophils from normal adults. The basal ROL of neutrophils depends heavily upon HSA concentration in the assay milieu[8]; however, the reproducibility of the basal mROL for a population of neutrophils from normal individuals is excellent.

To characterize the chemokinetic response of neutrophils to fMLP we linked the single cell assay to a flow-through system that allows (1) observation of the same neutrophils in a control and a response period, and (2) quantitative description of the locomotive behavior of a population of neutrophils and the individual cells that constitute the population. Previous studies have shown that neutrophils are not continuously in motion.[9,10,14] Their course of locomotion is interrupted by periods of inactivity as previously documented by Senda,[10] Ramsey,[14] and Howard.[9] Therefore, a neutrophil may increase its ROL in response to fMLP by increasing the rate of cell movement during periods when the cell is moving, by decreasing the amount of time the cell spends inactive, or a combination of the two. Conversely, a decrease in ROL in response to fMLP may be caused by an increase in inactive periods, a decrease in ROL when the cell is moving, or a combination of the two. We learned that the chemokinetic response of a population of neutrophils to 10^{-9} mol/L fMLP results from an increase in the active rate of locomotion of cells, ie, the cells move more rapidly when they are moving. The chemokinetic response to 10^{-9} mol/L fMLP does not reflect a decrease in the amount of time the cell spends inactive.

Also, we defined the fMLP dose dependency for the chemokinetic response of neutrophils exposed to serial increases in fMLP concentration (10^{-10} to 10^{-6} mol/L). The maximal mROL for responsive cells occurs at 10^{-9} mol/L fMLP and, as noted above, that increase in mROL reflects an increase in aROL, not a decrease in time spent inactive. In contrast, at fMLP concentrations $\geq 10^{-8}$ mol/L, the mROL decreased toward control values. This decrease is due to an increase in time inactive as well as a decrease in maROL; however, the increase in time inactive is the major change observed.

Examination of the chemokinetic response of individual cells reveals several points. First, the response of individual cells is highly variable. In fact, 17% to 21% of neutrophils within a population do not exhibit a chemokinetic response to 10^{-9} mol/L fMLP or any other concentration of fMLP. The most obvious locomotive characteristic of the nonresponsive cells is a rapid ROL ($\geq$14 um/min) prior to fMLP addition. The variability in chemokinetic response is consistent with heterogeneity of neutrophil function previously reported by our lab[9] and others.[9,19]

In addition to documenting the utility of single cell studies and quantitatively describing the chemokinetic response of neutrophils to fMLP, these studies yield information about how neutrophils change their rate of movement. The studies show that the neutrophil can alter its ROL either by changing its ROL when it is moving (aROL) or by changing the amount of time inactive as shown with cells exposed, by serial addition, to $\geq 10^{-7}$ mol/L fMLP. These observations heighten the potential importance of inactive intervals and raise questions about why they occur. We know the inactive intervals do not occur only at points where cells are executing acute turns.[9] Do the inactive periods represent a time for cytoskeletal reorganization, as probably occurs during sensory adaptation of neutrophils,[20] a period of ATP depletion following a burst of locomotion, or the time required to amass adequate numbers of assymetrically distributed fMLP receptors to allow the cell to advance?[21] Further study is necessary to explain inactive intervals. The clarification of conditions that increase inactive intervals of neutrophils will aid such studies.

Our observation that 21% of neutrophils are nonresponders matches the proportions of peripheral blood neutrophils which have previously been found to be deficient in a variety of cellular responses including chemotaxis.[19] However, no previous study has examined the chemokinetic response alone and none defined a characteristic by which the nonresponders can be identified without modifying the cell by treating with IgG, stimulants, or antibodies.[22,23] Our observation is that prior to fMLP stimulation the nonresponsive minority of cells move at a faster ROL than do responsive cells.

Finally, the data on fMLP dose dependence to serial addition of fMLP is of interest. First, compared to previous studies of fMLP dose dependency of chemokinesis, which were done with single additions of 10^{-10} mol/L or 10^{-9} mol/L or 10^{-6} mol/L fMLP to a single population of cells, the fMLP concentration that yields peak chemokinetic response is different.[18] With single addition the peak chemokinetic response occurred at 10^{-8} mol/L fMLP, while with serial addition peak response is observed at 10^{-9} mol/L fMLP. Therefore, the protocol used for fMLP addition affects the dose dependency of the chemokinetic response.

Serial addition of fMLP in the flow-through system demonstrates that variability in chemokinetic responsiveness among peripheral blood neutrophils is not limited to responders and nonresponders. Variability also occurs among responders with respect to optimal fMLP dose for maximal chemokinetic response. Twenty-seven percent of responsive neutrophils exhibit maximal chemokinetic response at 10^{-10} mol/L fMLP, and 25% require fMLP concentrations $\geq 10^{-8}$ mol/L or 10^{-7} mol/L, while the majority, 48%, respond to 10^{-9} mol/L fMLP. Even though the peak responsiveness is normally distributed around a mean of 10^{-9} mol/L, marked shifts in the relative proportions of responders and nonresponders or variations in the sensitivity of responders to stimulant may modify the kinetics of the cellular inflammatory response in vivo and render the host more or less susceptible to bacterial infection. Further studies are necessary to examine the relevance of alterations in the percentage of nonresponders and the sensitivity of responders to development of infection in man.

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Platelet Storage Pool Deficiency in Pigs

By Todd M. Daniels, David N. Fass, James G. White, and E. J. Walter Bowie

We report a new bleeding disease—storage pool deficiency (SPD) of platelets—in pigs from the Mayo swine colony of homozygous von Willebrand's disease (vWD) and of heterozygous carriers of vWD. Levels of factor VIII, von Willebrand factor antigen (vWF:Ag), and ristocetin cofactor (RCof) were similar in the vWD carriers and SPD pigs. The latter pigs, however, had bleeding times of 15 minutes or more and were severe bleeders, in contrast to clinically normal vWD carriers. Platelet aggregation in response to collagen was reduced in most SPD pigs. Total platelet content of ADP, ATP, and serotonin was less than that of normal pigs. While the initial uptake of ^{14}C-labeled serotonin into platelets was similar in SPD and normal pigs, retention of serotonin was reduced in platelets of SPD pigs. Transmission electron microscopy showed a large decrease of dense bodies in the platelets of SPD pigs. These findings support a diagnosis of SPD. Genetic analyses suggest an autosomal recessive mode of inheritance. A breeding program is under way to produce pigs affected only at the SPD gene, thus allowing further characterization of SPD and SPD-carrier pigs.

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IN 1967, a colony of swine affected by von Willebrand's disease (vWD) was established at the Mayo Clinic research facility from a single boar and three unaffected females.[1] Pigs homozygous at the Willebrand locus are characterized by trace levels of von Willebrand factor antigen (vWF:Ag) (0.025 U/dL) and ristocetin cofactor (RCof) (<3 U/dL), low levels of factor VIII, bleeding time of 15 minutes or more, and a severe bleeding tendency. Heterozygous (vWD carrier) pigs have low mean vWF:Ag and RCof values (26 and 30 U/dL, respectively), normal levels of factor VIII, and no bleeding tendency.[2,3] After several generations, a new bleeding manifestation became apparent. These "variant" pigs were characterized by levels of vWF:Ag, RCof, and factor VIII, similar to those seen in vWD carriers, but had bleeding times of 15 minutes or more and a severe bleeding tendency. Epistaxis, dental bleeding, and parturition were potentially life-threatening occurrences in these "variant" pigs, as in homozygous vWD pigs. Genetic analysis suggests that a new bleeding disorder, like porcine vWD, is transmitted in an autosomal recessive manner.[4]

Studies to characterize this bleeding disorder are reported here.

MATERIALS AND METHODS

Preparation of platelet-rich plasma and gel-filtered platelets. Blood was collected in 0.1 volume of 3.8% trisodium citrate or porcine intestinal mucosal heparin (Organon, West Orange, NJ) to a final concentration of 5 U/mL. Platelet-rich plasma (PRP) was prepared by centrifuging blood at 120 × g for 20 minutes. PRP to be assayed for platelet adenosine diphosphate (ADP), adenosine triphosphate (ATP), and serotonin was centrifuged for 4 minutes at 120 × g twice more to ensure the absence of red blood cells.

Citrated PRP (9 mL) was gel-filtered on a 2.5- by 20-cm 2% agarose column. Modified Tyrode's buffer[5] was used (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.36 mmol/L NaH_2PO_4, 1 mmol/L $MgCl_2$, 5 mmol/L HEPES, 5 mmol/L glucose, 0.38% trisodium citrate, 0.35% bovine serum albumin, pH 7 35), and 4-mL fractions were collected The platelets were pelleted and resuspended in normal, vWD, or storage pool deficiency (SPD) platelet-poor plasma (PPP) to a concentration of 2.5×10^5 platelets/μL.

Platelet aggregation. Aggregations were carried out in a Payton dual-channel aggregometer at 37 °C with continuous stirring at 800 rpm. Fifty microliters of an agonist was added to 450 μL of PRP or resuspended gel-filtered platelets (2.5×10^5 platelets/μL). ADP was used at final concentrations of 20 and 10 μmol/L. Bovine Achilles tendon collagen ("Type V," Sigma, St. Louis, Mo) was diluted in Tyrode's buffer (2 g/dL, Difco, Detroit, Mich) and homogenized intermittently in a Waring blender for 90 minutes at 0 °C. The supernatant was sonicated for 3 seconds before use, and was either used undiluted or mixed with an equal quantity of buffer.

ADP, ATP, and serotonin contents in platelets. Extracts were prepared from 7×10^8 platelets.[6] Lyophilized extracts were dissolved in 80 μL of 7 mmol/L potassium phosphate at pH 7.0 and filtered, and 5 to 20 μL was injected for analysis. Components for high pressure liquid chromatography consisted of a Beckman 450 data system/controller and two 114 M pumps, Waters model 441 UV detector at 254 nm, and a 0.46- by 25-cm anion exchange column (Whatman Partisil 10-SAX). ADP was eluted in 0.1 mol/L potassium phosphate at pH 4.2; ATP was eluted in a linear 0.4 to 0.5 mol/L phosphate gradient (ambient temperature, flow rate 3 mL/min) at 0.05 absorbance unit full scale (AUFS). Run time was 42 minutes. Serotonin was eluted with 0.01 mol/L phosphate at pH 3.1 at 1 mL/min. The 450 controller plotting factors were set at 30,000 for the nucleotides and 200,000 for serotonin. Standards were obtained from Sigma (St. Louis, Mo).

Uptake and retention of ^{14}C-labeled serotonin. A modification of the method of Weiss et al[7] was used. Five microliters of ^{14}C-labeled serotonin creatinine sulfate (Amersham, 55 mCi/mmol, 8 μCi/mL) was incubated in 2.5 mL of citrated PRP (6.25×10^8 platelets) for 15 minutes at 37 °C. Imipramine was then added to a final concentration of 10 μmol/L, and incubation was continued at 37 °C. Aliquots were removed at intervals and centrifuged, and the radioactivity of the supernatant was determined in a liquid scintillation counter (Beckman LS7500).

Transmission electron microscopy. Blood samples extracted by venipuncture were mixed immediately with citrate-citric acid-dextrose, pH 6.5 (93 mmol/L sodium citrate, 70 mmol/L citric acid, and 140 mmol/L dextrose) in a ratio of 9 parts blood to 1 part anticoagulant.[8,9] PRP was separated from whole blood by centrifugation at 100 × g.

Samples were combined with an equal volume of 0.1% glutaraldehyde in White's saline, pH 7.3—a 10% solution of a 1:1 mixture of (1) 2.4 mol/L NaCl, 0.1 mol/L KCl, 46 mmol/L $MgSO_4$, 64 mmol/L $Ca(NO_3)_2 \cdot 4H_2O$, and (2) 0.13 mol/L $NaHCO_3$, 8.4

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mmol/L $NaH_2PO_4 \cdot 7H_2O$, 3.8 mmol/L KH_2PO_4, and phenol red (0.1 g/L). After 15 minutes at 37 °C, the samples were sedimented to pellets, and the supernatant was discarded and replaced with 3% glutaraldehyde in the same buffer. Fixation was continued at 24 °C for 60 minutes. The cells were then washed in buffer and combined with 1% osmic acid in barbital acetate buffer (0.02 N HCl, a 20% stock solution containing 0.14 mol/L sodium barbital and 0 145 mol/L sodium acetate, and a 6 8% solution of a stock solution containing 1.7 mol/L NaCl and 18 mmol/L $CaCl_2$) After exposure to the second fixative for 1 hour, the cells were dehydrated in a graded series of alcohol and embedded in an epoxy resin (Epon 812). Contrast of thin sections cut from plastic blocks on an ultramicrotome was enhanced with uranyl acetate and lead citrate. Observations were made with an electron microscope (Philips 301).

Infusion studies Two SPD swine were placed on their backs in wooden cradles and restrained by ropes; pigs lie quietly in this position. One 136-kg pig received 1,400 mL of normal pig cryoprecipitate prepared by the method of Slichter et al.[10] The other SPD pig was infused with 410 mL of normal pig platelet concentrate prepared by plateletphoresis in a cell processor (Haemonetics model 30 blood cell processor). The total platelet content was 10.7 × 10^{11}, which was calculated to result in an increase of 3.0 × 10^8 platelets/mL. Blood was removed at intervals for hemostatic tests, and ear bleeding times were determined.

RESULTS

Aggregation studies. Collagen-induced aggregation in citrated PRP was absent or decreased in 10 of the 11 SPD pigs (Fig 1). The response to collagen of platelets from vWD and vWD-carrier pigs was varied (two of eight vWD pigs and two of six vWD-carrier pigs having absent or decreased collagen-induced aggregation). A likely explanation for this finding is that some vWD-carrier pigs were heterozygous at the SPD gene and the vWD pigs were either homozygous or heterozygous at the SPD gene. A breeding program is under way to breed separate strains of SPD and vWD pigs and to characterize pigs of each of the nine possible genotypes involving the SPD and vWD genes.

Results of studies using resuspended gel-filtered platelets are plotted in Fig 2. The maximal recorder pen deflection was expressed as ΔT. SPD platelets were less responsive to collagen whether resuspended in normal, vWD, or autologous PPP. In contrast, normal platelets aggregated in response to collagen when resuspended in PPP from pigs with SPD.

ADP, ATP, and serotonin contents of platelets. The total content of adenine nucleotides, as well as serotonin, in SPD platelets was less than that of normal pigs (Fig 3). Mean values in SPD pigs studied were 31% for ADP, 34% for ATP, and 23% for serotonin of the mean values in normal pigs. To test whether the low values in SPD pigs were

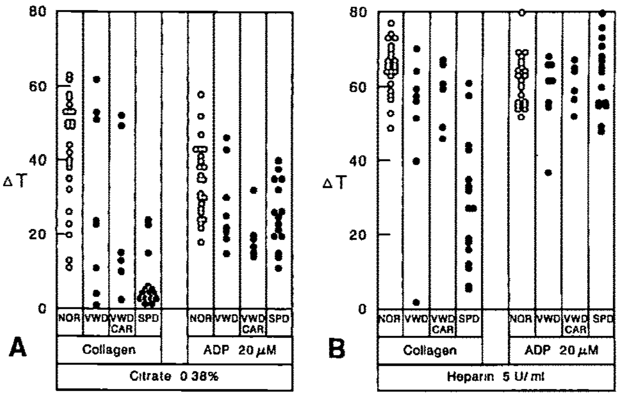

Fig 1. Aggregation response of platelets of pig to collagen or ADP expressed as maximal ΔT within 5 minutes of addition of agonist. Fifty microliters of agonist was added to 450 μL of citrated (A) or heparinized (B) PRP adjusted to 2.5 × 10^8 platelets/mL. NOR, unrelated normal pigs; VWD, pigs with homozygous von Willebrand's disease; VWD CAR, heterozygous carriers of von Willebrand's disease; and SPD, storage-pool-deficient pigs.

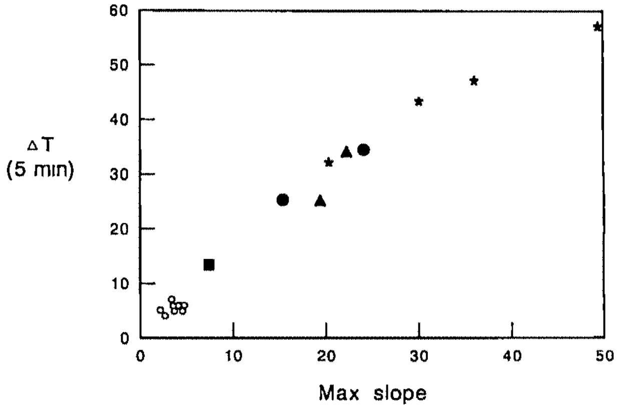

Fig 2. Aggregation response of reconstituted gel-filtered platelets to collagen, plotted as maximal ΔT within 5 minutes *v* maximal slope. Citrated normal or SPD pig PRP was gel-filtered on a 2% agarose column equilibrated in modified Tyrode's buffer (see Materials and Methods). Platelets were pelleted and resuspended to 2.5 × 10^8 platelets/mL with normal, SPD, or vWD pig PPP. ▲, normal gel-filtered platelets in normal plasma; ■, normal gel-filtered platelets in vWD plasma; ●, normal gel-filtered platelets in SPD plasma; *, unfractionated normal PRP control; O, SPD gel-filtered platelets in normal, vWD, or SPD plasma, and unfractionated SPD PRP control.

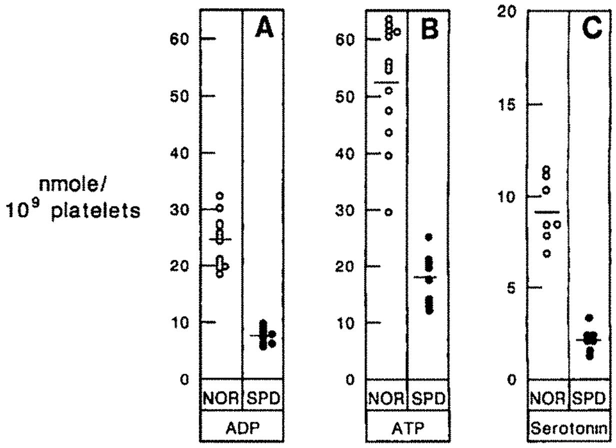

Fig 3. (A) ADP, (B) ATP, and (C) serotonin contents of unrelated normal and SPD pig platelets. Each platelet pellet (7 × 10^8 platelets) was freeze-thawed, extracted twice with 12% trichloroacetic acid, extracted 10 times with diethyl ether, and lyophilized. Samples were dissolved in 80 μL of phosphate buffer, and 5 to 20 μL was injected for analysis by HPLC. Phosphate buffers were used to elute sample components from an analytical anion exchange column, and absorbance at 254 nm was monitored.

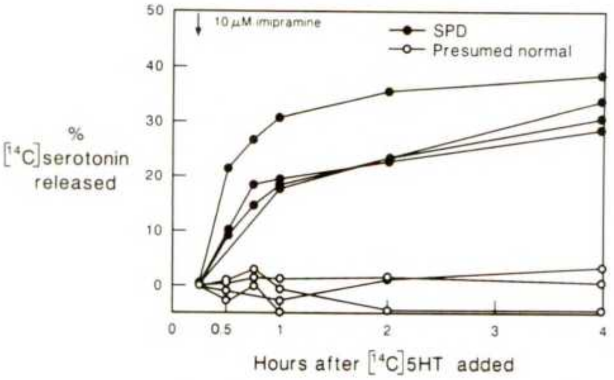

Fig 4. Release of ^{14}C-labeled serotonin from SPD and unrelated normal pig platelets. ^{14}C-labeled serotonin (0.29 µmol/L final concentration) was added to 2.5 mL of citrated PRP (2.5 × 10^8 platelets/mL). After 15 minutes at 37 °C, imipramine was added to a final concentration of 10 µmol/L, and incubation at 37 °C was continued. Aliquots (300 µL) were removed at intervals and centrifuged, and supernatant radioactivity was determined in a liquid scintillation counter.

characteristic of swine in our closed herd, ADP and ATP contents were determined in three vWD pigs and serotonin content was determined in one vWD pig. Pigs with vWD whose platelets aggregated normally in response to collagen were used for these determinations. Mean values were ADP 109.8%, ATP 104.7%, and serotonin 91.8% of the mean values for unrelated normal pigs.

Uptake and retention of ^{14}C-labeled serotonin. Uptake of ^{14}C-labeled serotonin after 15 minutes was similar in the two groups of pigs (normal 76.2%, SPD 70.7%). At 15 minutes, imipramine was added to inhibit the reuptake of any serotonin released by the platelet. Release of ^{14}C-labeled serotonin from SPD platelets was evident 15 minutes after the imipramine was added; and at 4 hours, SPD platelets had released approximately one third of the ^{14}C-labeled serotonin originally taken up (Fig 4). Normal pig platelets retained the amine for 4 hours.

Electron microscopic findings. Thin sections of the platelet preparation from a normal and a SPD pig were examined by transmission electron microscopy. There were 57 dense bodies in thin sections of 125 platelets in the normal pig and 15 dense bodies in the same number of cells from the SPD pig (Fig 5).

Infusion studies. Enough normal porcine cryoprecipitate was infused into a SPD pig to raise the levels of vWF:Ag from 26 to 97 U/dL and of RCof from 29 to 103 U/dL 30 minutes after infusion. The bleeding time remained abnormal (over 15 minutes) through 24 hours (Fig 6). Such an infusion corrects the bleeding time in vWD pigs.[11] Another SPD pig received platelet concentrate from a normal pig, which raised its platelet concentration by a maximum of 2.1 × 10^8 platelets/mL 1 hour after infusion. Bleeding time was shortened and transiently corrected to normal (Fig 6). Increased collagen-induced platelet aggregation was apparent after platelet infusion (Fig 6). Before infusion, the response curve to collagen had a slope of 1.5 and a zero ΔT. (This can occur because SPD platelets undergo a transient change in shape, resulting in a measurable slope; however, ΔT does not exceed zero—the original baseline—in some instances.)

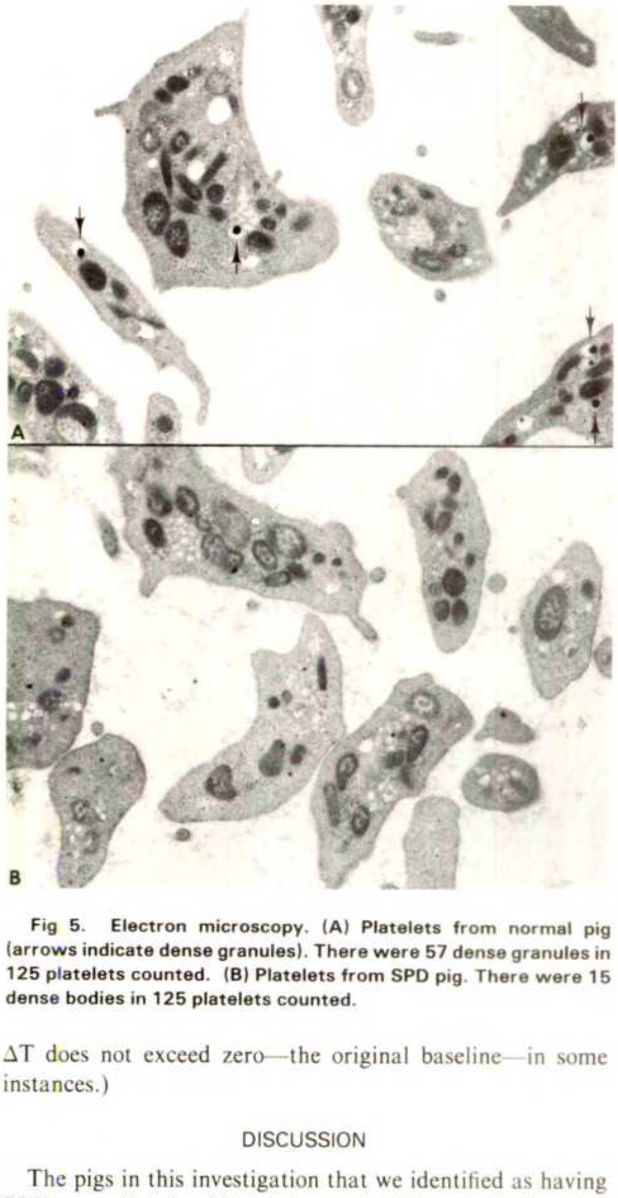

Fig 5. Electron microscopy. (A) Platelets from normal pig (arrows indicate dense granules). There were 57 dense granules in 125 platelets counted. (B) Platelets from SPD pig. There were 15 dense bodies in 125 platelets counted.

DISCUSSION

The pigs in this investigation that we identified as having SPD were all derived from a herd of vWD and vWD-carrier swine. Our studies have shown that the platelets of these SPD pigs have a decreased number of dense granules and decreased amounts of serotonin, ADP, and ATP. Furthermore, platelet retention of serotonin after the addition of imipramine was impaired.

Pigs with homozygous vWD are characterized in part by extremely low vWF:Ag and RCof values (less than 3 U/dL), bleeding times of more than 15 minutes, and a life-threatening bleeding diathesis. Pigs heterozygous for vWD have higher, but not normal, mean levels of vWD-Ag and RCof (26 and 30 U/dL, respectively), normal bleeding times, and no bleeding tendency. Pigs from our herd with SPD have vWF:Ag and RCof levels similar to those in pigs heterozygous for vWD but, in contrast, have prolonged bleeding times—often more than 15 minutes—and a life-threatening bleeding tendency.

The observation that the SPD pigs originated in a vWD herd prompted us to investigate the possibility that they were

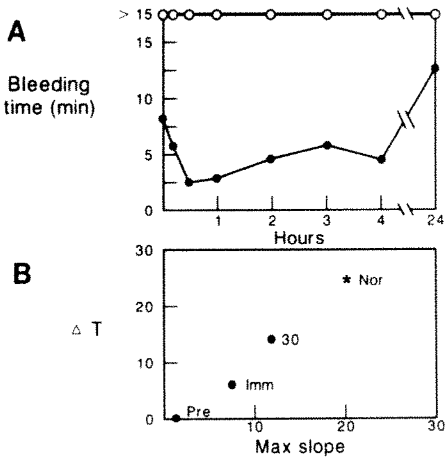

Fig 6. Infusion of cryoprecipitate or platelet concentrate of normal pig into SPD pigs. (A) Solid circles represent bleeding times before and after infusion of 410 mL of platelet concentrate from normal pig. Platelet count increased by a maximum of 2.1×10^8 platelets/mL at 30 minutes after infusion. Open circles represent bleeding times before and after infusion of 1,400 mL of normal porcine cryoprecipitate. FVIII, vWF:Ag, and RCof increased by 37%, 273%, and 255% over preinfusion levels, respectively, 30 minutes after infusion. Each bleeding time is the mean of two determinations per time point per pig. In the pig, mean (± SD) normal bleeding time is 2.2 ± 0.75 minutes and vWD carrier bleeding time is 3.2 ± 1.3 minutes.[4] Time of cessation of initial bleeding was used; "rebleeding," a phenomenon common in SPD pigs, is not indicated in the graph. (B) ΔT *v* maximal slope of collagen-induced platelet aggregation curve before and after infusion of platelet concentrate. Pre, preinfusion; Imm, immediately after infusion; 30, 30 minutes after infusion; and Nor, unrelated normal pig control.

affected by a variant form of vWD similar to that found in humans because they had long bleeding times and mean values of vWF:Ag and RCof of 25 and 35 U/dL, respectively. Examination of the von Willebrand factor multimers, however, revealed no abnormality, and our experience with porcine vWD suggested that the plasma level of von Willebrand factor was not low enough to cause bleeding without some additional abnormality being present. This led us to investigate other causes of the prolonged bleeding time and to the discovery of platelet SPD.

Like vWD, SPD is inherited as a simple autosomal recessive locus segregating independently of the similarly autosomal recessive von Willebrand locus.[4] The vWD pigs in our herd, therefore, are homozygous, heterozygous, or normal at the SPD locus. Those with homozygous SPD display impaired collagen-induced platelet aggregation; all others have normal collagen-induced aggregation and nucleotide content. The vWD carriers also are either heterozygous or normal at the SPD locus. The vWD carriers have normal bleeding times and therefore cannot be homozygous at the SPD locus. Pigs with prolonged bleeding times, low platelet nucleotide content, decreased collagen-induced aggregation, and mean levels of vWF:Ag and RCof of 25 and 35 U/mL, respectively, are SPD pigs that also happen to be carriers of vWD. We have not been able to identify SPD pigs that definitely do not carry vWD. The lower values of vWF:Ag and RCof in normals overlap the higher values in carriers.[3,4]

Because our breeding program was focused on producing vWD and vWD-carrier pigs, it can be expected that most of the SPD pigs identified were carriers of vWD. In fact, pedigree analysis shows that all of the SPD pigs in this study may be carriers of vWD (eg, some SPD pigs were the offspring of one vWD pig, and the others were offspring of vWD carriers). We have initiated a backcross breeding program to produce pigs affected only at the vWD locus or the SPD locus. It will then be possible to determine if the decreased levels of vWF:Ag and RCof are due to heterozygosity at the vWD locus or are linked to the expression of SPD.

Like platelets in the human disease, platelets in porcine SPD show abnormal aggregation with collagen. The secondary response of platelet aggregation to epinephrine and ADP is also abnormal in human SPD. Using our aggregation procedure, however, platelets of normal pigs do not aggregate in response to epinephrine, and there is only primary (reversible) aggregation with ADP. Thus, abnormal collagen-induced aggregation is the only detectable abnormality of porcine platelet aggregation that resembles SPD in humans.

Platelet storage pool deficiency in humans can be categorized on the basis of a decrease of substances stored in the dense granules (SPD or δ-SPD), rarely the α-granules (α-SPD or gray platelet syndrome), or both dense and α-granules ($\alpha\delta$-SPD).[12,13] SPD involving dense granules often accompanies other clinical abnormalities, such as the Chédiak-Higashi syndrome,[14,15] Hermansky-Pudlak syndrome,[16,17] Wiskott-Aldrich syndrome,[18] and thrombocytopenia absent radii syndrome.[19]

SPD has been found in association with the Chédiak-Higashi syndrome in mink,[20] cattle,[21] and cats.[22] Several pigment mutations in mice have been associated with SPD.[23,24] Recently, it has been shown that SPD in fawn-hooded rats[25,26] is a pleiotropic effect of the red-eyed dilution gene.[27]

The only additional abnormality that we have thus far detected in our SPD pigs is reduced vWF:Ag and RCof. We believe that the original breeding strategy would have produced many SPD pigs that were also carriers of vWD, so that pigs produced by the current breeding program and affected only at the SPD gene may have normal levels of vWF:Ag and RCof. If this is true, these pigs may be an animal model for the idiopathic inherited SPD found in humans.[7,12,28,29]

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Expression of Normal Myeloid-Associated Antigens by Acute Leukemia Cells

By Patricia A. Dinndorf, Robert G. Andrews, Denis Benjamin, Derry Ridgway, Lawrence Wolff, and Irwin D. Bernstein

Monoclonal antibodies that react with hematopoietic cells and their precursors in a stage and lineage restricted fashion were used in indirect immunofluorescence assays to examine leukemic cells from 105 pediatric age patients. The differentiative states of blasts from 42 patients with acute nonlymphocytic leukemia (ANLL) were defined by these antibodies. When these were compared to their morphologic and histochemical levels of differentiation as defined by the French-American-British (FAB) classification, no direct relationship was found. The reactivity of these antibodies with leukemic cells from 63 patients with acute lymphocytic leukemia (ALL) was also investigated, and the usefulness of these antibodies in distinguishing leukemias of myeloid from those of lymphoid origin was demonstrated.

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ACUTE nonlymphocytic leukemia (ANLL) is a heterogeneous group of disorders that originates at the level of hematopoietic progenitor cells. ANLL subtypes have classically been categorized according to morphologic and histochemical criteria, which have been more precisely defined in recent years by the French-American-British (FAB) classification.[1] An alternate approach to assess the heterogeneity of ANLL is to use monoclonal antibodies that react with antigens expressed by normal hematopoietic cells and their precursors in a lineage and stage-restricted fashion.

We have used such monoclonal antibodies to examine the differentiative state of ANLL in children.[2,3] Importantly, a comparison of the differentiative state of ANLL cells based on morphologic and histochemical criteria (defined by the FAB classification), with their antigenic phenotype failed to reveal a direct relationship. In addition, we have used these antibodies to examine acute lymphocytic leukemia (ALL) cells, and demonstrated their usefulness in distinguishing leukemias of myeloid versus lymphoid origin.

MATERIALS AND METHODS

Cells Heparinized marrow or peripheral blood obtained at the time of the initial diagnosis or relapse of leukemia was obtained from pediatric patients at Children's Orthopedic Hospital and Medical Center, Seattle, Washington, and at the Doernbecher Children's Hospital, Oregon Health Sciences University, Portland. Samples from children with ANLL were collected beginning in 1973 at the Children's Orthopedic Hospital and Medical Center, and at the University of Oregon beginning in September 1979 when the Children's Cancer Study Group (CCSG) 251 study for the treatment of ANLL was opened. ALL samples were collected beginning in January 1979 with the inception of the CCSG study 191 for the treatment of average and high risk ALL, and from Oregon beginning in May 1983 with the inception of CCSG protocols 104, 105, 106, 107, and 123 for the treatment of children with various risk factors. Cells were separated by Ficoll-Hypaque density centrifugation (density 1.077), suspended in newborn calf serum supplemented with 10% dimethyl sulfoxide (DMSO), and frozen in liquid nitrogen until studied. All samples had greater than 75% malignant blast cells, as determined by examination of Wright stained cytospin preparations of thawed cells at the time of phenotyping, and viability was greater than 75% as assessed by trypan blue dye exclusion.

Monoclonal antibodies. Unfractionated ascites fluids containing monoclonal antibodies were used at dilutions from 1:250 to 1:1,000 in all studies. Antibody T11D7, an isotype identical (IgM) murine monoclonal antibody of irrelevant specificity (antimouse Thy 1.1; kindly provided by Dr E Clark, University of Washington, Seattle) served as a negative control. Production and characterization of monoclonal antibodies 1G10, 5F1, L4F3, and T5A7 has been described elsewhere[2-6] and their reactivities are summarized in Table 1. Monoclonal antibodies to lymphoid-associated antigens used to characterize ALL included CALLA (J-5 provided by Dr John Pesando or BA-3 provided by Dr John Kersey)[10] and T-cell antibodies (9.6, 10.2, 12 1, and 38.1 provided by Dr John Hansen and 3A1 provided by Dr Barton Haynes).[11]

Immunofluorescent studies Cells were incubated with monoclonal antibody diluted in Minimal Essential Medium (MEM) supplemented with 5% newborn calf serum and 2% human AB serum (Irvine Scientific, Santa Ana, Calif) for 30 minutes, washed, and then incubated with a 1:40 dilution of an affinity-purified fluorescein-conjugated goat antimouse IgM antiserum (Litton Bionetics, Kensington, Md) as previously described[2] As a negative control, cells also were stained with the isotype identical antibody, T11D7 (antimouse Thy 1.1). Stained cells were analyzed by flow microfluorimetry using a FACS 440 (Becton Dickinson, Oxnard, Calif). A total of 4,000 cells was analyzed for each antibody. Antibody staining of cells was considered positive if more than 25% of cells exhibited fluorescence intensity greater than 95% of the cells stained with the negative control antibody. Twenty five percent was used as the cutoff because all samples analyzed contained no more than 25% normal contaminating cells and, therefore, at this level at least some of the reactivity was with leukemic cells. In the case of L4F3, if there was a homogeneous shift of the median fluorescence intensity (peak) to greater than 1.5 times that of cells stained with T11D7, cells were also considered positive. In cases where there was a homogeneous shift, 100% of the cells were considered to express the antigen. This conclusion was confirmed by demonstrating that for 23 leukemic samples that had been determined to express L4F3 by these criteria, all cells were susceptible to complement-mediated lysis by L4F3 in a ^{51}Cr release assay. The complement-mediated cytotoxicity assay has been previously described.[2]

Cytochemical studies and FAB classification. The diagnosis of ALL or ANLL was made at the institution of origin based on standard morphological and cytochemical criteria. Leukemia classified as ALL was not peroxidase, sudan black, or chloracetate esterase positive; most were PAS positive. Marrows of patients

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Table 1. Monoclonal Antibodies to Myeloid-Associated Antigens

Antibody	Biochemic Specificity	Reactivity With Hematopoeitic Cells and Precursors	Equivalent Antibodies
1G10	X-hapten[5] Galβ1-4GlcNAcR 3 Fucα1	PB: granulocytes (bright) 10–15%, monocytes (barely detectable staining) BM: myelocytic cells past myeloblast stage; portion of myeloblasts Progenitor cells: portion CFU-GM[3]	Multiple [CD 15]* antibodies[7]
5F1	85 Kd[4] glycoprotein	PB: monocytes, platelets BM: monocytes, nucleated erythroid cells, megakaryocytes Progenitor cells CFU-E[3]	20.3[8] [CDw14]*
L4F3	62 Kd† protein	PB: monocytes and granulocytes (weak) BM. immature myelocytes, monocytes Progenitor cells: CFU-GM, portion of BFU-E, CFU-Meg, CFU-Mix[3,28]	MY-9[9] L1B2[3]
T5A7	lactosylceramide[6]	PB: granulocytes, monocytes BM. mature granulocytic cells and some band forms (bright), band forms and metamyelocytes (moderately bright) Progenitor cells: portion Day 7 CFU-GM[3] proportion PHA-stimulated lymphocytes	G035[7] [CDw17]*

Abbrev: PB, peripheral blood; BM, bone marrow

*Differentiation clusters as defined by 2nd International Workshop on Human Leucocyte Differentiation Antigens

†Bernstein I, Andrews RG: unpublished observations.

diagnosed as having ANLL were reviewed by two investigators (DB, PAD) specifically examining cytochemical stains including Wright-Giemsa, periodic acid Schiff (PAS), peroxidase, sudan black, NSE (alpha naphthyl butyrate), chloracetate esterase, and acid phosphatase. These cases were classified according to the FAB schema.[1] That is, cases that were peroxidase or sudan black positive were categorized as M1, M2, or M3, depending on the degree of differentiation. Cases that were peroxidase (sudan black) and NSE positive were classified as M4. Specimens that were NSE positive only were classified as M5. Leukemia was classified as M6 or erythroleukemia based on the morphologic appearance of cells and PAS reactivity.

Terminal deoxynucleotidyl transferase (TdT) staining. TdT determinations were made on cryopreserved leukemic cells of the patients whose cells expressed myeloid antigens. Cytospin preparations were fixed in absolute methanol at 4 °C for 30 minutes, then incubated with rabbit anti-TdT serum, followed by FITC conjugated goat $F(ab')_2$ fragments specific for rabbit IgG (Bethesda Research Laboratories, Gaithersburg, Md).

RESULTS

Reactivity of monoclonal antibodies with ANLL cells. The reactivity of monoclonal antibodies 1G10, 5F1, T5A7, and L4F3 with leukemic samples from 42 pediatric patients with ANLL was examined by indirect immunofluorescence. The specificities of these antibodies and antibodies that appear to recognize the same determinants are summarized in Table 1. In brief, two of these antibodies discriminate between granulocytic and monocytic cells. 1G10, an X-hapten reactive antibody (CD15),* reacts with cells at all stages of granulocytic differentiation to the promyelocyte level, but only with a portion of myeloblasts and granulocyte-macrophage progenitors (CFU-GM). In contrast, antibody 5F1 reacts with monocytes, platelets, megakaryocytes, nucleated erythrocytes, and erythroid colony-forming cells (CFU-E), but not with granulocytic cells.

The other two antibodies, L4F3 and T5A7, react with antigens expressed at maximal concentrations on granulocytic cells at different stages of maturation. Thus, L4F3 reacts most intensely with metamyelocytes, myelocytes, promyelocytes, and a portion of myeloblasts, and virtually all CFU-GM, while the T5A7 antigen (lactosylceramide) is expressed maximally on mature granulocytes, and a minor proportion of Day 7 CFU-GM. Both of these antibodies also react with monocytes. In addition, T5A7 reacts with 20% of PHA-stimulated lymphocytes.

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*Designation of the 2nd International Workshop on Human Leucocyte Differentiation Antigens.[7]

The results show that 40 of 42 leukemic samples examined reacted with at least one of the antibodies (see Fig 1). 1G10, which predominantly reacts with granulocytes, bound to 55% of the samples tested, while cells from 69% of the samples bound 5F1, the monocyte reactive antibody. Thirty-five specimens (83%) reacted with either one or both of these antibodies. Ninety percent of the samples tested reacted with L4F3, the antibody that binds maximally to immature myeloid cells. Cells from 61% of the samples reacted with T5A7, the antilactosylceramide antibody that binds maximally to mature myeloid cells. Only two specimens (5%) failed to react with any of the antibodies tested.

FAB classification of ANLL cells. For each patient studied, bone marrow aspirates obtained at initial diagnosis were stained with Wright Giemsa, and for peroxidase, sudan black and NSE, and were classified according to the FAB classification for ANLL. Cells from 15 patients were classified as M1 (myeloblast), 4 as M2 (myeloid with maturation), 1 as M3 (promyelocytic) (confirmed by the 15:17 chromosomal translocation), 16 as M4 (myelomonocytic), 4 as M5 (monocytic), and 2 as M6 (erythroblastic). The M5 category was subdivided into 2 cases of M5A (immature) and 2 cases of M5B (differentiated).

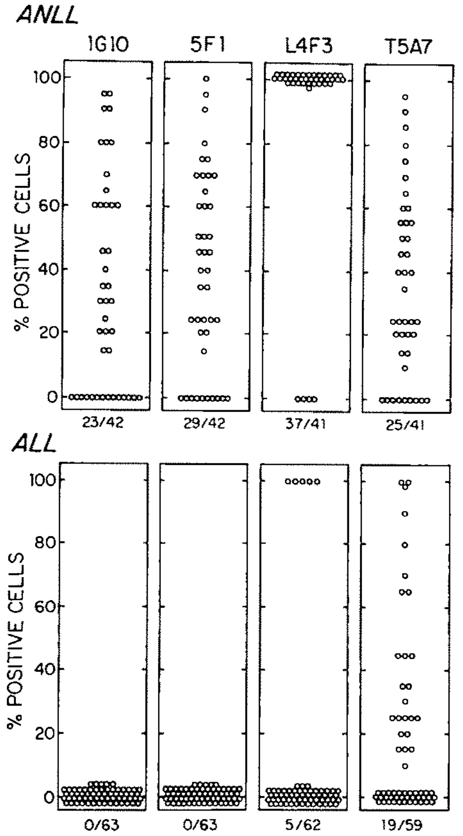

Fig 1. Reactivity of monoclonal antibodies with ANLL and ALL. The reactivity of leukemic cells with monoclonal antibodies was determined for each specimen by indirect immunofluorescent antibody binding and analysis by flow microfluorimetry. For each antibody a specimen was considered positive if ≥25% of cells were reactive. [(No. specimens positive)/No. specimens tested) = (percent of specimens positive)].

Comparison between antigenic phenotype and FAB classification of ANLL cells. The differentiative state of leukemic cells defined according to FAB classification or antigenic phenotype were generally discordant (Table 2). First, most specimens classified as FAB M1 did not react with 1G10 which reacts with most granulocytic elements. This may reflect the immaturity of these cells with respect to granulocytic differentiation. However, leukemic cells which appeared by conventional criteria to have differentiated further along the granulocytic pathway, ie, specimens classified as M2 and M3, also failed to react with 1G10 in three of five instances. In contrast, the majority of these cells (M1, M2, and M3) reacted unexpectedly with 5F1, a reaction not seen with normal granulocytes. Also unexpected was the reaction of all leukemias classified as FAB M5 with 1G10. One pattern that did emerge was that specimens classified as FAB M4 tended to react with all the antibodies tested.

The relationship between maturation according to antigenic and morphologic criteria was also evaluated. Since the surface expression of the antigen identified by L4F3 is greatest on the least as compared to the most mature myeloid cells,[3] we determined whether this relationship was also found between leukemic cells classified as less or more differentiated. A comparison of the relative fluorescence intensity of samples stained with L4F3 among the FAB subgroups revealed no obvious relationship between fluorescence intensity and maturation (data not shown). This however may have been a consequence of the small number of more mature (M2, M3, M5b) specimens. A similar lack of a relationship between morphologic and antigenic differentiation was not found for T5A7 which reacts maximally with mature myeloid cells. Seven of 15 leukemias classified as M1 (myeloblast) reacted with T5A7, while only one of five samples that were morphologically more mature and classified as M2 or M3 reacted with T5A7.

Table 2. Reactivity of Monoclonal Antibodies With Subgroups of ANLL

	Antibodies			
FAB/Cytochemistry	1G10	5F1	L4F3	T5A7
M1/peroxidase				
(Immature myeloid)	3/15	10/15	14/15	7/15
M2/peroxidase				
(Myeloid with maturity)	2/4	2/4	2/4	1/4
M3/peroxidase				
(Promyelocytic)	0/1	0/1	ND	0/1
M4/peroxidase, NSE				
(Myelomonocytic)	14/16	12/16	16/16	13/16
M5A/NSE				
(Monocytic, immature)	2/2	1/2	1/2	2/2
M5B				
(Monocytic, mature)	2/2	2/2	2/2	1/2
M6				
(Erythroblastic)	0/2	2/2	2/2	1/1

Number of samples reacting with antibody over number of samples tested of each FAB subclassification.

Comparison between peripheral blood and bone marrow phenotype of ANLL cells. Because peripheral blood rather than marrow cells was often evaluated, we compared antigenic phenotypes of leukemic cells obtained from the peripheral blood and marrow in eight patients in whom both types of specimens were available. These paired samples were obtained at the same time in the patient's disease, that is, at initial diagnosis or at the time of a subsequent relapse. Importantly, the proportion of leukemic cells reactive with the antibodies 1G10 and 5F1 as determined in peripheral blood or bone marrow was similar (within 15%). In all instances, except for one patient each in the case of antibody 5F1, 1G10, and T5A7, the positive or negative nature of the bone marrow or peripheral blood sample was concordant.

In studies with T5A7, a slightly greater percent (<15% except in one instance where it was 25%) of leukemia cells were positive in the peripheral blood as compared to the marrow in almost every case. Again these differences altered the interpretation of T5A7 reactivity in only one case. This may have been the consequence of a greater number of normal mature cells contaminating the peripheral blood specimens or a higher proportion of the circulating malignant cells as compared to those in the marrow which express lactosylceramide in the periphery. The former, however, is unlikely in the absence of a consistently greater activity of 1G10 or 5F1 with peripheral blood granulocytes or monocytes, respectively, that might have been present in the peripheral blood specimens.

Expression of myeloid-associated antigens by ALL cells. Leukemic samples from 63 pediatric patients with ALL were also examined for reactivity with the anti myeloid monoclonal antibodies (see Fig 1). These patients were diagnosed as having ALL based on morphological and cytochemical criteria. The reactivity of these cells with lymphoid-associated monoclonal antibodies was reported in a previous study[12] and is summarized in Table 3. None of the specimens reacted with either antibody 1G10 or 5F1. Lymphoblasts from 5 of 62 samples tested reacted with L4F3. Of the five, three expressed CALLA and the other two lacked both CALLA and T-cell antigens. Clinically, these five patients all underwent remission induction successfully and remain in complete continual remission 38, 53, 54, 59, and 68 months from initial diagnosis.

Table 3. Reactivity of Monoclonal Antibodies With Subgroups of ALL

Serologically Defined Subgroups	Myeloid Antibodies			
	1G10	5F1	L4F3	T5A7
T+* CALLA + or −	0/16	0/16	0/15	8/15
T− CALLA +	0/41	0/41	3/41	7/38
T− CALLA −	0/6	0/6	2/6	4/6

Indirect immunofluorescent reactivity of monoclonal antibodies with malignant cells by FACS analysis. For each antibody a specimen was considered positive if ≥25% of cells were reactive. Results are reported as (number of samples positive)/(number of samples tested).

*Specimens were tested with either antibodies 9.6 and 10.2 separately or with a cocktail consisting of 9.6, 10.2, 12.1, 3A1, and 38.1.

Cells from six of the patients with ALL did not express the T-cell–associated antigens that were evaluated or CALLA. Cells from two of these patients reacted with L4F3 as mentioned above, and one reacted with both L4F3 and T5A7. Of the three cases in this group that reacted only with T5A7, two were infants who died within one year of diagnosis, an outcome typical of this disease in infants. The third remains in continuous remission at 31 months. The patient whose cells failed to react with any of the antibodies tested also remains in initial remission at 23 months.

The antigen defined by T5A7, lactosylceramide, was expressed on a significant proportion of lymphoblasts in 19 of 59 specimens tested. Eight of 15 T-cell leukemias tested expressed this antigen. This is not surprising, since T5A7 is known to react with a proportion of PHA-stimulated peripheral blood T cells.[3] Four of eight patients with T-cell ALL whose blasts reacted with T5A7 remain in complete continual remission from 25 to 54 months from initial diagnosis while three of seven patients whose cells did not react with T5A7 are alive 26 to 61 months from initial diagnosis. Cells of seven of 38 CALLA positive cases were reactive with T5A7. However, there appeared to be no difference in this small series in the survival of patients whose cells did and did not react with T5A7 (data not shown).

Terminal deoxynucleotidyl transferase (TdT) determinations were done on all but two of the ALL specimens which stained with one or more antimyeloid antibodies. Of the five that were L4F3 positive, four were tested for TdT expression, and all were positive; and of the 19 specimens that reacted with T5A7, 18 were tested and all were positive.

DISCUSSION

ANLL is generally categorized according to morphological and histochemical criteria set forth by the FAB group and is based on expression of characteristics normally associated with granulocytic, monocytic, or erthrocytic cells.[1] This classification has largely failed to identify groups of prognostic importance. This system relies on subjective criteria and concordance between hematopathologists is not high. We have directly compared the assignment of cell lineage defined by the FAB system to that defined by reactivity with antimyeloid monoclonal antibodies. In the present study, a comparison of differentiative states of leukemic cells defined by the presence of histochemical markers versus that defined by the expression of myeloid-associated antigens failed to demonstrate a direct relationship between these two. Specifically, an association between the presence of peroxidase and the expression of a granulocyte-associated antigen (1G10) was not found. Nor did we observe a direct correlation between the presence of NSE and the expression of a monocyte-associated antigen (5F1).

This failure to define a relationship between lineage assignments defined by these two methods is not surprising since each of these approaches examines the expression of only a few of the gene products normally displayed by myeloid cells of specific lineages or at specific stages of maturation. Thus, in the FAB system only cytoplasmic peroxidase and NSE, which are selectively expressed during normal granulocytic or monocytic differentiation, respectively, are evaluated. Examination of the expression of cell surface differentiation antigens thus provides evidence of other maturation-linked characterizations that can be evaluated to assess the differentiative state of ANLL cells.

Evaluation of the morphologic appearance of cells also did not strictly correlate with the antigenic phenotype. An immature appearance (FAB-M1 cell) was not necessarily associated with expression of an antigen predominantly found on immature granulocytes (L4F3) nor was a more mature appearance (FAB M3) associated with expression of an antigen found on mature granulocytes (T5A7). There were, however, two FAB subclasses that demonstrated distinct patterns of antigenic expression. Specifically, most of the specimens of FAB M1 ANLL did not express the antigen detected by 1G10 (12 of 15). This antigen is expressed by virtually all granulocytic cells except the least mature myeloblasts and CFU-GM (where only a portion are 1G10 positive). In the second instance FAB M4 specimens frequently reacted with all of the antibodies used in the present study, consistent with the multiple expression of cytoplasmic enzymes detected by histochemic methods. Nevertheless, analysis of cell surface antigens provides an independent assessment and can provide a novel basis for the classification of patients with ANLL.

A number of other investigators have examined the expression of myeloid antigens on ANLL cells.[15-22] Herrmann et al[17] demonstrated quantitative differences in the number of ANLL cells expressing antigens in the various subgroups defined by the FAB classification. One of the monoclonal antibodies used was VIMD5, which also reacts with the X-hapten.[23] Analogous to our results with 1G10, they found the X hapten on only 15% to 30% of the FAB M1 leukemias tested, which was a lower proportion than that found for the other FAB subclasses, except for M6.

Griffin et al[20] defined patterns of antigen expression with a series of monoclonal antibodies that correspond to discrete levels of differentiation. They observed patterns that corresponded to some FAB groups, that is, M3 and M5, but these patterns were not exclusively expressed by leukemias of these subtypes, and the number of patients in these groups was small. Similar to our results with 5F1, MY-4, an antibody that reacts with monocytes, was expressed by some samples that were NSE negative. This is in contrast to a study in which monoclonal antibodies were described that reacted with nearly every case of FAB M4 and M5 leukemia tested.[21]

Of potential interest is that antigenic phenotyping of ANLL with these antibodies may identify groups of prognostic importance. The FAB classification has been only minimally successful in identifying groups of clinical relevance. In a preliminary report by Vaughan et al[24] a correlation between successful remission induction and reactivity with MY-1, another antibody to the X-hapten, was reported.[25] In contrast, Griffin et al[20] failed to detect a relationship between antigenic phenotype and probability of remission induction. Unfortunately, the patients in the present study with ANLL were collected over a 10-year period and were not treated in a uniform fashion and therefore a clinical analysis is not possible. We are, however, presently using these antibodies to study a larger number of pediatric patients in a prospective manner who are enrolled in clinical protocols of the Children's Cancer Study Group.

A subclassification of ANLL that is not accounted for in the FAB schema is acute megakaryoblastic leukemia. One patient who was not included in the present series experienced a classical course of acute megakaryblastic leukemia.[26] Leukemic cells from this patient failed to react with any of the antibodies used in the present study although they did react with C7E10, an antibody prepared in our laboratory to glycoprotein Ib.[27] Nineteen specimens of ANLL included in the present study were found not to react with C7E10 (data not shown).

In the present study we also examined leukemic cells of lymphoid origin and observed that antibodies 1G10, 5F1, and L4F3 are potentially useful in differentiating ALL from ANLL. Ninety-five percent of the cases of ANLL tested reacted with at least one of these three antibodies. Eighty-three percent of the ANLL samples tested reacted with either 1G10 or 5F1 or both. None of the 63 cases of ALL tested reacted with either of these antibodies. Thus, these antibodies are potentially useful in cases where the differentiation between these diseases is not straightforward. In a previous report, we identified two such patients who were diagnosed as having ALL although their leukemic cells reacted with 1G10 and/or 5F1. These patients were subsequently reclassified as ANLL, based on a change in morphology in one patient and in the clinical course in the other. Cells from these two patients failed to express CALLA, B-cell, or T-cell antigens.[12]

Reactions of antimyeloid antibodies with cells that were unequivocally of lymphoid origin were also observed in a number of instances. Antibody L4F3, which reacted with 90% of the ANLL specimens tested also reacted with 8% of the ALL samples tested. Similarly, Griffin et al have reported that MY-9 reacted with 90% of ANLL specimens, and with 2% of ALL specimens obtained from adults.[9] Studies in this laboratory have shown that L4F3 and MY-9 may detect the same antigen as determined by competitive binding-inhibition studies (unpublished observation, R. Andrews). Furthermore, antibody T5A7 reacted with 32% of the ALL samples tested, especially with T cell leukemias. This reactivity with T-cell leukemias is not surprising since T5A7 is known to react with PHA-stimulated lymphocytes. The evaluation of additional patients with ALL who express these antigens is required to assess the clinical significance of these unexpected expressions of myeloid-associated antigens on leukemic cells of lymphoid origin.

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Monoclonal Antibodies Binding to the Human Neutrophil C3bi Receptor Have Disparate Functional Effects

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Three monoclonal antibodies (MAb)—OKMI, 7C3, and 60.3—immunoprecipitated a common 170-kd neutrophil membrane antigen closely associated with, or identical to, the C3bi receptor (CR3). Despite binding to a common receptor, these antibodies displayed marked differences in their effects on C3bi-mediated neutrophil function as assessed by the binding and ingestion of opsonized zymosan and the subsequent triggering of the respiratory burst. Antibody 7C3 caused a time-dependent, irreversible inhibition of the neutrophil oxidative response to opsonized zymosan that correlated with capping of the bound antibody. In contrast, antibody 60.3 caused an immediate inhibition of the neutrophil oxidative response to opsonized zymosan that required the continuous presence of exogenous antibody to achieve the maximal inhibitory effect. Antibody OKMI demonstrated minimal inhibition of O_2^- release. Despite their functional differences, binding of either 7C3 or 60.3 led to up-regulation of new antigen, presumably from intracellular sites as previously described using OKMI. Crossed immunoprecipitations of radiolabeled neutrophil lysates indicated that each MAb bound to different antigens near or within the CR3 complex. Thus three MAb binding to the neutrophil CR3 receptor each caused receptor up-regulation but had markedly different functional effects on the cell.

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RECEPTORS for the complement fragment C3bi (CR3) located on the surface membrane of human neutrophils mediate the binding and ingestion of particles coated with this opsonin, and therefore are critically involved in cellular host defense.[1,2] The development of monoclonal antibodies (MAb) directed against this receptor has provided the opportunity to investigate the consequences of alterations in receptor number and activity. We compared two recently described MAb, 7C3[3,4] and 60.3,[5,6] previously reported to inhibit C3bi-mediated neutrophil function, to investigate the cellular response to antibody-mediated receptor blockade. The effects of these MAb were compared with those induced by OKM1, a third MAb known to bind to the neutrophil C3bi receptor.[7] The relationships among these antibodies were further elucidated by the biochemical characterization of the antigens immunoprecipitated by these antibodies.

MATERIALS AND METHODS

Monoclonal antibody production, purification, and creation of F(ab')$_2$ fragments Antibody 60.3 (IgG$_{2a}$) was produced as previously described [5] Antibody for experimental use was collected as ascites fluid from pristane-primed mice that had been inoculated with approximately 10^6 hybridoma cells. After purification using affinity chromatography with protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden),[8] 60.3 was aliquoted and stored at −70 °C before experimental use. For designated experiments the antibody was directly fluoresceinated with fluorescein isothiocyanate (FITC; Research Organics, Cleveland),[9] or radioiodinated with ^{125}I (New England Nuclear, Boston) using the iodogen method.[10] Antibody concentration was determined using the Bradford assay.[11]

Antibodies 7C3 (IgG$_3$) and 31D8 (IgG$_1$), kindly provided by Dr Harry Malech, Yale University, New Haven, Conn, were produced as described.[3] Antibody 7C3 was recloned to the single-cell level by limiting dilution before use. Antibody was collected from murine ascites fluid, purified using protein A-Sepharose,[8] and stored at −70 °C Antibodies 7C3 and 31D8 were linked to FITC or ^{125}I as described for antibody 60.3. Antibody OKM1 (IgG$_{2b}$)[7] was obtained from Ortho Diagnostic Systems, Raritan, NJ, purified using Sephacryl S-200, and conjugated with FITC or ^{125}I as described for the other antibodies. MAb 31D8 binds to neutrophils at a site distinct from the C3bi receptor (D.H., unpublished observation, November 1984); this MAb served as a control antibody in designated experiments.

F(ab')$_2$ fragments of MAb 7C3 and 60.3 were produced after incubation with immobilized pepsin (Pierce Chemical Co, Rockford, Ill) for two hours at 37 °C at pH 4 5 [12] Protein A-Sepharose was used to absorb F$_c$ fragments and residual intact immunoglobulins. The purity of the eluted F(ab')$_2$ fragments was established after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions.

Neutrophil preparation. Peripheral blood was obtained from normal volunteers in accordance with institutional protocols, or as leukopaks obtained from normal donors during plateletpheresis at Puget Sound Blood Center, Seattle Neutrophils were isolated by dextran sedimentation at 37 °C either directly or after density-gradient centrifugation through Ficoll-Hypaque (Histopaque; Sigma Chemical Co, St Louis).[13] Residual erythrocytes were removed by hypotonic lysis. Cells were washed and resuspended in Hanks' balanced salt solution (HBSS) at the desired concentration as determined by the neutrophil number, viability, and differential count. The purified neutrophil fraction contained 85% to 98% polymorphonuclear cells (PMNs) when isolated after Ficoll-Hypaque and 65% to 85% PMNs without this initial step. Viability was always greater than 97% as assessed by trypan blue exclusion.

Binding of monoclonal antibodies to PMNs. Saturating concentrations of each antibody were determined by incubating 5 × 10^5 neutrophils with increasing dilutions of MAb at 4 °C for 30 minutes in round-bottom microtiter wells (Linbro, Flow Laboratories, McLean, Va) in a final volume of 100 μL HBSS. Cells were washed twice with HBSS at 4 °C to remove unbound antibody and incubated at 4 °C for 30 minutes with 25 μL of a 1:50 dilution of affinity-purified, fluorescein-conjugated, sheep antimouse F(ab')$_2$ antiserum

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(Cappel, Cochranville, Pa). After two further washes, cells were fixed with 1% paraformaldehyde in normal saline at 4 °C for 15 minutes.

Immunofluorescence of these neutrophils was quantitated using an Ortho 50 H cytofluorograph interfaced to a model 2150 Computer (Ortho Diagnostic Instruments, Westwood, Mass). Fluorescent excitation used a wavelength of 488 nm; emission was measured at 515 to 575 nm. The fluorescent signal was log amplified by selection of the photomultiplier setting. Neutrophils were gated selectively from lymphocytes and monocytes on the basis of right- and forward-angle light scatter. The mean fluorescent intensity for the neutrophil population at each antibody dilution was measured and plotted on logarithmic paper. The point of departure from the plateau of the curve was determined to be the minimum saturating concentration. The dilution of each antibody used experimentally was at least twofold higher than the minimal saturating dose. Using these criteria, the following amounts of antibody were required per 5×10^5 cells: antibody 3ID8, 0.75 µg; antibody 60.3, 2 µg; antibody 7C3, 0.75 µg; antibody OKMI, 0.4 µg.

In the assessment of temperature and time of incubation on PMN-associated fluorescence, PMNs were incubated with saturating concentrations of FITC-linked antibodies at 4 °C for 30 minutes, washed free of unbound antibody, and incubated at 4 °C or 37 °C for five or 30 minutes before fixation. Mean fluorescence of the PMN population was quantitated by flow cytometry. The percentage of maximal fluoresence of the cell population was obtained by converting the log fluorescent values to linear values, subtracting the control cell values, and expressing the result as a percentage of the maximal linear fluorescence. In direct immunofluorescence experiments, the maximal fluorescence was that obtained by neutrophils exposed to a fluorescent first antibody and not subjected to further experimental manipulation

In the indirect immunofluorescence assay, PMNs were incubated with saturating concentrations of antibody at 4 °C for 30 minutes, washed free of unbound antibody, and incubated at 4 °C or 37 °C for five or 30 minutes before fixation and incubation with the FITC-labeled second antibody. After washing, the mean fluorescence of the cell population was quantitated by flow cytometry. The percentage of maximal fluorescence was calculated as described for the direct immunofluorescence assay, except that the maximal fluorescence referred to that of neutrophils exposed to the MAb and not subjected to further experimental manipulation before incubation with the FITC-labeled second antibody.

In co-capping studies, 5×10^6 PMNs were incubated simultaneously in microtiter wells at 4 °C or 37 °C for 30 minutes with saturating concentrations of MAb 7C3 and FITC-60.3 or FITC-OKM1 After fixation and washing to remove unbound antibody, cells were examined using fluorescent microscopy. These studies were repeated using $F(ab')_2$ fragments of MAb 7C3 and FITC-60.3 or FITC-OKM1.

The effect of MAb 7C3 on the binding of the other two antibodies was assessed in two separate experiments. PMNs (5×10^5) were incubated with saturating concentrations of MAb 7C3 or $F(ab')_2$ fragments of 7C3 at 4 °C for 30 minutes and then incubated at 4 °C or 37 °C for 30 minutes before fixation and incubation with FITC-60.3 or FITC-OKM1. PMNs were washed to remove unbound antibody and examined using both fluorescent microscopy and flow cytometry.

For direct studies using iodinated antibodies, ^{125}I-labeled antibody (50,000 cpm) was incubated with 5×10^5 cells at 4 °C for 30 minutes in a total volume of 100 µL in 96-well microtiter plates (Costar, Cambridge, Mass). Cells were washed and incubated at 4 °C or 37 °C for zero or 30 minutes before centrifugation through 300 µL of a suspension of dinonyl phthalate (one part) and dibutyl phthalate (four parts) silicon oil. After removal of the supernatant silicon oil, the cell-bound radioactivity remaining in the pellet was determined. Nonspecific ^{125}I-antibody binding was determined by saturating the cells with a 100-fold excess of unlabeled MAb before exposure to the ^{125}I-labeled antibody. Specific antibody binding was calculated by subtracting values for nonspecific binding from those obtained with cells incubated with ^{125}I-labeled antibody alone.

The indirect ^{125}I assay was performed by incubating 5×10^5 cells with saturating concentrations of MAb at 4 °C for 30 minutes, washing, and subsequently resuspending and incubating the cells at 4 °C or 37 °C for zero or 30 minutes. Cells were then fixed with paraformaldehyde and incubated with ^{125}I-labeled sheep antimouse $F(ab')_2$ (50,000 cpm). After 30 minutes, cells were centrifuged through silicon oil as described, and the residual radioactivity in the pellet was counted. Values for nonspecific ^{125}I-antibody binding were obtained by incubating cells with the second antibody alone. These values were subtracted from those measured for MAb-coated cells to determine specific binding.

Quantitation of antigen binding sites was performed using ^{125}I-labeled $F(ab')_2$ fragments of MAb 60.3 and 7C3. Human PMNs (2×10^6 cells) were incubated with increasing concentrations of labeled antibody in the absence or presence of 100-fold molar excess of unlabeled antibody. After incubation for 60 minutes at 4 °C, PMNs were separated from unbound antibody by centrifugation through 300 µL of phthalate oil as described in the direct iodination studies. Radioactivity remaining in the pellet was used to calculate specific binding, the amount of antibody bound, and the bound/free ratio was determined. The number of antibody binding sites per PMN was estimated using Scatchard analysis [14]

Conditions of MAb binding for functional studies. Two conditions for MAb binding were examined in parallel. Under "antibody present" conditions, 5×10^5 PMNs were incubated with antibody at 37 °C for ten minutes and membrane stimulants were then added directly to the reaction mixture. In the "antibody removed" conditions, PMNs were incubated with antibody (4 °C for 30 minutes), washed at 4 °C to remove unbound antibody, and warmed to 37 °C over ten minutes before membrane stimulation. Thus exogenous antibody was present at the time of stimulation under "antibody present" conditions but was removed before stimulation under "antibody removed" conditions. Control conditions examined concurrently included identically handled PMNs that had not been exposed to antibody or that had been incubated with control monoclonal antibody 31D8.

Stimuli. Zymosan particles (Sigma) were preopsonized with pooled human serum that had been stored undiluted at −70 °C after collection. Opsonized particles were prepared daily after incubation at 37 °C for 30 minutes in 50% human serum in HBSS. Zymosan was washed and resuspended in HBSS before use A single batch of zymosan was used for all experiments. Controls were performed with the same zymosan preparation each day. Zymosan particles prepared in this manner did not bind FITC-labeled MAb 60.3, 7C3, OKM1, or 31D8. Phorbol myristate acetate (PMA; Consolidated Midland Corp, Brewster, NY) was dissolved in dimethyl sulfoxide at 1 mg/mL and stored at −70 °C until use. FITC was coupled to immunological grade zymosan (ICN Nutritional Biochemicals, Cleveland) as described.[15] The FITC-zymosan was aliquoted and stored at −20 °C before opsonization on the day of use with pooled human serum as described

Superoxide release. Superoxide (O_2^-) release was measured directly in Linbro flat-bottomed, 96-well microtiter plates (Flow Laboratories) using a modification of the Pick and Mizell[16] microassay for superoxide dismutase (SOD; Sigma)-inhibitable ferricytochrome c (Sigma) reduction. The microtiter wells were first incubated with 100 µL gelatin (0.5 mg/mL) to decrease the O_2^- release by unstimulated neutrophils and washed with HBSS before use. The reaction mixture contained 5×10^5 neutrophils prepared under the conditions described above, 10.9 nmol ferricytochrome c and 20 μL MAb with or without 20 μg SOD in a final volume of 100 μL HBSS. MAb were used in concentrations previously demonstrated to saturate binding sites on PMNs. Either PMA (100 ng/mL) or serum opsonized zymosan (particles: cells = 10:1) were added in 10-μL vol. After incubation at 37 °C for 20 minutes on a microshaker (setting 3; Dynatech Laboratories, Alexandria, Va), the change in the light absorption at OD 550 was measured using a Titertek Multiscan (Titertek; Flow Laboratories). Total reducable ferricytochrome c was determined after adding 10 μL of a saturated sodium dithionate solution to each well and remeasuring the OD 550. For each time point the SOD-inhibitable ferricytochrome c reduced was calculated as a percentage of the total dithionite-reducable ferricytochrome c and multiplied by 10.9 nmol to calculate the total nmol of O_2^- released. Due to variation in the calculated extinction coefficient for reduced ferricytochrome c at different concentrations of the compound, this method was demonstrated to be most reproducible. In preliminary experiments, none of the MAb interfered directly with ferricytochrome c reduction. Interference with transmission of the light signal by zymosan in the O_2^- assay was corrected by subtraction of the SOD-containing wells used as a blank.

H_2O_2 release. Release of H_2O_2 was measured by the oxidation of scopoletin as previously described.[17]

Determinations of cell-associated particles. After preparation with MAb under the "antibody present" or "antibody removed" conditions as described, 5 × 10^5 PMNs were incubated in gelatin-blocked Linbro microtiter plates at 37 °C with continuous agitation with opsonized FITC-zymosan (particles: cells = 10:1) in 100 μL HBSS. After 20 minutes, cells were fixed with 1% paraformaldehyde, washed twice, and transferred to glass slides for examination by fluorescence microscopy. The number of zymosan particles within the borders of 100 consecutive PMNs was recorded.

Samples prepared in parallel were assessed for cell-associated fluorescence by flow cytometry The threshold fluorescence intensity used was that at which 99% of the total cell population not exposed to FITC-zymosan was negative. With flow cytometry, control neutrophils demonstrated a mean fluorescence of less than or equal to 20; PMNs with a fluorescence greater than or equal to 80 on a log scale contained cell-associated, FITC-linked zymosan. This fluorescence intensity represented a tenfold increase in brightness when at least one FITC-zymosan was associated with a neutrophil. Prolonged incubation (longer than 30 minutes) of PMNs with FITC-zymosan was associated with a mild decrement in cell-associated fluorescence. This did not adversely affect the assay, since the intensity of fluorescence of the labeled zymosan permitted reliable detection despite mild attenuation of the signal.

Immunoprecipitation and gel electrophoresis. PMNs (5 × 10^7 cells) were surface labeled with ^{125}I using a modification of the lactoperoxidase procedure.[18] After cell lysis by incubation in 0.5% NP40, 0.15 mol/L NaCl, 0.05 mol/L Trizma, and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF; Sigma), immunoprecipitations were performed using 50 μg purified MAb coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). In the crossed immunoprecipitation experiments, the cell lysates were extensively precleared with the "preclearing antibody" before the addition of the precipitating antibody. One-dimensional SDS-PAGE was performed under reducing conditions according to the method of Laemmli.[19]

Statistical analysis. The paired *t* test was used to assess the statistical significance of the results shown in the designated figures and tables.

RESULTS

Inhibition of C3bi-mediated PMN respiratory burst activity by MAb. The comparative ability of these antibodies to inhibit O_2^- release was measured to relate these studies to previously reported findings (Table 1).[3] Monoclonal antibody 31D8, not known to affect C3bi function, was used as an antibody control. Although all three antibodies, 7C3, 60.3, and OKM1, demonstrated an effect on PMN respiratory burst activity triggered by opsonized zymosan, the extent of inhibition and the conditions required for the maximal inhibitory effect were markedly different. Antibody 7C3 exerted its major effect when exogenous antibody was removed after 30 minutes' preincubation with PMNs. When exogenous 7C3 antibody was present during the assay, O_2^- release was inhibited only slightly compared with control antibody-treated PMNs. In contrast, antibody 60.3 markedly suppressed zymosan-triggered O_2^- release when antibody was present. When 60.3 was removed after preincubation with cells, O_2^- release increased to levels approaching control values. Antibody OKM1 had a modest inhibitory effect on zymosan-generated O_2^- release that was only observed in the presence of exogenous antibody. Similar results were obtained when H_2O_2 release, rather than O_2^-, was measured (data not shown). None of these antibodies significantly affected O_2^- release in response to PMA over 20 minutes. None of the antibodies were cytotoxic under the conditions used as assessed by trypan blue exclusion.

When these experiments were repeated using $F(ab')_2$ fragments, antibody 60.3 inhibited O_2^- release to levels almost identical to those observed with the intact MAb, ie, O_2^- release was inhibited to 38.5% of control when the $F(ab')_2$ fragment was present (n = 3) and to 78.3% of control when the $F(ab')_2$ fragment was removed after preincubation (n = 3). Inhibition of O_2^- release by the $F(ab')_2$ fragment of MAb 7C3 followed the pattern demonstrated for the intact antibody. The antibody fragment inhibited O_2^- release to 76% of control when antibody was present (n = 4) and to 64% of control when antibody was removed after preincubation (n = 6).

Table 1. Effect of MAb on Superoxide Release by Human PMNs

Control (27)	PMA 7.3 ± 0.2	Opsonized Zymosan 2.0 ± 0.18
Antibody Present	% Control	
3ID8 (5)	93.9 ± 2.4	93.7 ± 5.8
7C3 (4)	98.0 ± 2.7	86.4 ± 5.6
60.3 (13)	99.2 ± 3.4	34.7 ± 5.4*
OKMI (9)	98.5 ± 3.9	81.0 ± 8.0†
Antibody Removed		
3ID8 (5)	94.9 ± 2.1	97.6 ± 5.0
7C3 (4)	106.0 ± 2.1	62.8 ± 2.6†
60.3 (13)	97.3 ± 2.0	79.1 ± 6.3
OKMI (9)	95.6 ± 1.8	104.0 ± 7.7

Superoxide release was measured over 20 minutes by SOD-inhibitable ferricytochrome c reduction as described in Materials and Methods. Values shown are the mean ± SEM for the number of experiments shown in parentheses. Values for controls represent nmol $O_2^-/5 \times 10^5$ PMN/20 min with the number of experiments shown in parentheses. Unstimulated control neutrophils released 0.72 ± 0.13 nmol $O_2^-/5 \times 10^5$ PMN/20 min

*$P < .005$ when compared with antibody 3ID8.

†$P < .025$ when compared with antibody 3ID8.

Kinetics of antibody-mediated interference with PMN respiratory burst activity. The incubation times required for antibodies 7C3 and 60.3 to exert their inhibitory effects on zymosan-triggered O_2^- release were investigated (Fig 1). PMNs were incubated with saturating concentrations of antibody at 37 °C for 0, 10, 20, or 30 minutes before opsonized zymosan was added to the reaction mixture. Superoxide generation was assayed 20 minutes after the addition of zymosan. The decrease in O_2^- release by PMNs incubated with 7C3 was linearly related to the time of exposure of neutrophils to 7C3 before stimulation. In contrast, incubation of antibody 60.3 with neutrophils produced an immediate, profound inhibition of O_2^- release that did not change by prolonging the duration of antibody exposure to neutrophils before stimulation. Temperature during preincubation did not appear to be a critical factor, since PMNs incubated with 7C3 at 4 °C before the addition of zymosan had comparably reduced O_2^- release.

Cell-associated particles. Neutrophils were prepared with the designated antibodies as described and then incubated with opsonized, FITC-linked zymosan (Table 2). The number of cell-associated particles was determined by both direct microscopic and flow cytometric assays. As with the O_2^- assay, the maximal inhibitory effect of 7C3 required a 30-minute preincubation of cells with antibody. In contrast, 60.3 exhibited an immediate inhibitory effect that required the presence of antibody in the reaction media for maximal inhibition. Antibody OKM1 displayed a slight inhibitory effect under "antibody present" conditions.

Kinetics of antibody binding. To determine whether the differences in time of onset of action of 7C3, as compared with 60.3 and OKM1, were related to differences in antibody binding rates, the kinetics of antibody binding were assessed by flow cytometry. Using directly fluoresceinated 7C3, 60.3, or OKM1, each antibody achieved complete saturation of binding to neutrophils within 15 minutes at both 4 °C and 37 °C (data not shown). Incubation of cells with saturating concentrations of 60.3 or OKM1 did not inhibit the binding of 7C3 or of each other, as assessed by flow cytometry.

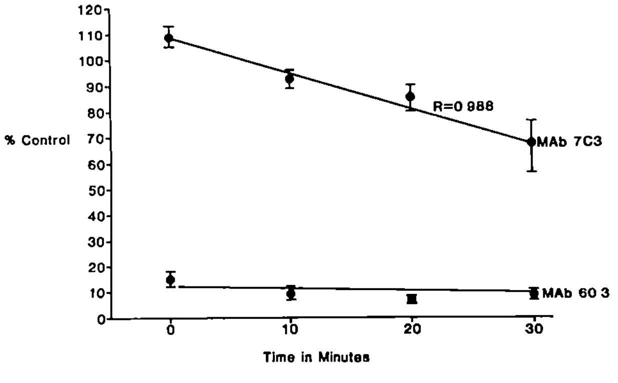

Fig 1. Effect of time of exposure of neutrophils to MAb on subsequent ability to trigger O_2^- release by opsonized zymosan. Time of exposure (shown on the abscissa) refers to the duration of incubation of MAb with neutrophils before the addition of opsonized zymosan. All assays were read 20 minutes after the addition of the stimulus. Control PMNs released 2.2 ± 0.3 nmol of O_2^- per 5×10^6 PMNs per 20 minutes. For 7C3, the r value equaled 0.988.

Table 2. Influence of MAb on Cell Association of FITC-Linked Opsonized Zymosan With PMN

	Cell-Associated Particles (%)	
	Microscopic	Flow Cytometry
Antibody Present		
Buffer (6)	62.3 ± 12.5	45 ± 4.6
Antibody 31D8 (6)	67.0 ± 7.8	50 ± 4.1
Antibody 60.3 (6)	34.2 ± 11.0*	16 ± 2.4†
Antibody 7C3 (6)	52.3 ± 13.7	42 ± 3.8
Antibody OKMI (3)	50.0 ± 1.1	ND
Antibody Removed		
Buffer (6)	53.7 ± 9 3	50 ± 1.4
Antibody 31D8 (6)	53.3 ± 8.9	52 ± 1.2
Antibody 60 3 (6)	57.7 ± 11.1	45 ± 1 4
Antibody 7C3 (6)	37.5 ± 17.6*	37 ± 1.2*
Antibody OKMI (3)	59.3 ± 4.5	ND

Microscopic percentage of cell association was obtained by counting 100 consecutive PMNs and determining the percentage of cells containing one or more zymosan particles. Flow cytometry percentage of cell association was determined by assessing the percentage of PMNs with a fluorescent intensity indicating that one or more zymosan particles was PMN associated. Data represent the means ± SD for the number of experiments shown in parentheses. ND, not done.

*$P < .05$.

†$P < .01$.

Detection of cell-associated antibody. Directly fluoresceinated 60.3 was used to determine whether the recovery of function by 60.3 pretreated cells after washing could be related to a loss of cell-associated 60.3. Comparative studies were carried out with FITC-7C3, FITC-OKM1, and FITC-31D8. Neutrophils were preincubated with saturating concentrations of FITC-MAb at 4 °C for 30 minutes, washed free of unbound antibody, and then incubated at 4 °C or 37 °C for five or 30 minutes before fixation and quantitation of fluorescence by flow cytometry.

The mean fluorescence of neutrophils incubated at 4 °C after exposure to each FITC-antibody did not differ significantly from that of cells fixed immediately after incubation with antibody (Table 3). Incubation of FITC-7C3–treated neutrophils at 37 °C resulted in a prompt drop in the fluorescence intensity by five minutes, which remained constant at 30 minutes. After warming to 37 °C, the fluorescence of FITC-60.3– or FITC-OKM1–treated neutrophils decreased slowly to 80% of maximal by 30 minutes. Neutrophils exposed to FITC-31D8 and warmed to 37 °C demonstrated no loss of cell-associated fluorescence.

Detection of PMN surface-associated antibody. Indirect immunofluorescence was investigated to determine whether the antibody remained associated with the cell surface after antibody binding and warming (Table 4). After PMNs were incubated at 4 °C with saturating concentrations of antibody, cells were washed and maintained at either 4 °C or 37 °C for five or 30 minutes before fixation and indirect immunofluorescence. The results paralleled those observed with direct immunofluorescence. There was no significant decrement in the mean fluorescence of any of the cell populations when incubations were carried out at 4 °C

Table 3. Effect of Temperature and Time of Incubation on PMN-Associated Fluorescence After Binding of FITC-Linked MAb

	Monoclonal Antibody (%)			
Incubation Time	31D8	60.3	7C3	OKMI
4 °C for 0 min	100	100	100	100
4 °C for 5 min	99.0 ± 2.1	92.0 ± 1.7	97.1 ± 1.5	90.3 ± 4.3
4 °C for 30 min	95.0 ± 3.9	93.5 ± 2.6	97.0 ± 1.2	97.6 ± 1.1
37 °C for 5 min	122.0 ± 4.6	95.5 ± 1.4	56.2 ± 4.0*	104.7 ± 6.8
37 °C for 30 min	111.0 ± 2.6	80.5 ± 6.1	54.6 ± 7.3†	83.9 ± 6.1

Results are expressed as the percentage of maximal fluorescence, where 100% equaled the fluorescence of PMNs preincubated with FITC-antibody and fixed at zero minutes (immediately after washing away unbound antibody). Values shown are mean ± SEM for three experiments.

*$P < .01$ when compared with results at 4 °C.

†$P < .05$ when compared with results at 4 °C.

for five or 30 minutes. However, at 37 °C the fluorescence saturation of 7C3-treated cells dropped to less than 50% by 30 minutes. In contrast, 60.3 and OKM1 demonstrated only a modest decrement in surface antibody after incubation at 37 °C for 30 minutes. These studies of cell- and surface-associated antibody were confirmed in studies using iodinated MAb (data not shown).

Morphological changes in PMNs incubated with 7C3 or 60.3. After incubation with FITC-7C3 or FITC-60.3, PMNs were warmed on a glass microscope slide and examined by fluorescence microscopy. When cells were maintained at 4 °C, both antibodies displayed a uniform, rim appearance on the cell. With warming for 30 minutes, however, more than 90% of cells incubated with 7C3 displayed significant capping of the antigen (Fig 2). In contrast, less than 10% of cells treated with 60.3 displayed capping after 30 minutes. Results similar to those observed for antibody 60.3 were seen using FITC-OKM1 (data not shown).

When PMNs were directly incubated with MAb 7C3 and FITC-OKM1 or FITC-60.3 at 4 °C or 37 °C, the fluorescence did not redistribute into the polar cap. Instead, the fluorescence remained circumferentially distributed at both temperatures. This pattern appeared with both intact MAb 7C3 and the $F(ab')_2$ fragment of 7C3.

Up-regulation of antigens. Whereas the time-related effect of 7C3 on PMN dysfunction correlated with antibody-induced membrane capping, the mechanism by which neutrophils recovered function after removal of exogenous 60.3 remained unclear. For these experiments PMN were incubated at 4 °C with saturating concentrations of 60.3 that blocked the subsequent binding of FITC-60.3 to cells maintained at 4 °C. Warming these cells to 37 °C resulted in the rapid reappearance of 60.3 surface antigen, as detected by the binding of newly added FITC-60.3 to the surface of the warmed cells (Fig 3). If PMNs were stimulated with either PMA or opsonized zymosan (100:1 particle to cell ratio) after exposure to the blocking antibody but before exposure to FITC-60.3, the amount of surface antigen increased to one and one half times that seen in PMNs not exposed to the stimuli. Comparable results were obtained using ^{125}I-60.3 antibody (data not shown).

Because up-regulation of the 60.3 antigen seemed to correlate with the recovery of PMN responsiveness to C3bi-coated particles, experiments were performed using antibodies 7C3 and OKM1. Surprisingly, each of these antigens was up-regulated in a similar fashion (Fig 3). Although most of the binding of FITC-60.3, FITC-OKM1, and FITC-7C3 was due to the appearance of "new" antigenic sites, some of this binding reflected the appearance of "old" sites from which the cold antibody had dissociated It was more difficult to blockade 7C3 binding sites completely, presumably reflecting the relative ease with which antibody 7C3 was shed (Table 3 and Table 4). When the control antibody 31D8 was used, an increase in antigen binding sites was not observed relative to cells warmed to 37 °C without additional stimulation.

Because of the morphological changes induced by 7C3, we examined the capacity of the PMNs to up-regulate the 60.3 and OKM1 binding sites after incubation with 7C3. In two separate experiments, PMNs saturated with MAb 7C3 at 4 °C and warmed to 37 °C up-regulated FITC-60.3 and

Table 4. Effect of Temperature and Time of Incubation on PMN Surface-Associated Antibody After Binding Using Indirect Immunofluorescence

	Monoclonal Antibody (%)			
Incubation Time	31D8	60.3	7C3	OKMI
4 °C for 0 min	100	100	100	100
4 °C for 5 min	103 ± 1.4	101 ± 0.7	97.6 ± 2.0	98.5 ± 2.5
4 °C for 30 min	99 ± 0.7	100	101.0 ± 0.7	94.5 ± 3.9
37 °C for 5 min	103 ± 6.1	86 ± 3.9	60.9 ± 3.3*	64.0 ± 2.1*
37 °C for 30 min	82 ± 5.0	83 ± 1.4	48.5 ± 2.8*	65.5 ± 3.2*

Results are expressed as the percentage of maximal fluorescence, where 100% equaled the fluorescence of PMNs preincubated with antibody, fixed at zero minutes (immediately after washing away unbound antibody), and immediately incubated with the fluorescent second antibody. Values shown are mean ± SEM for three experiments.

*$P < .01$ when compared with results at 4 °C

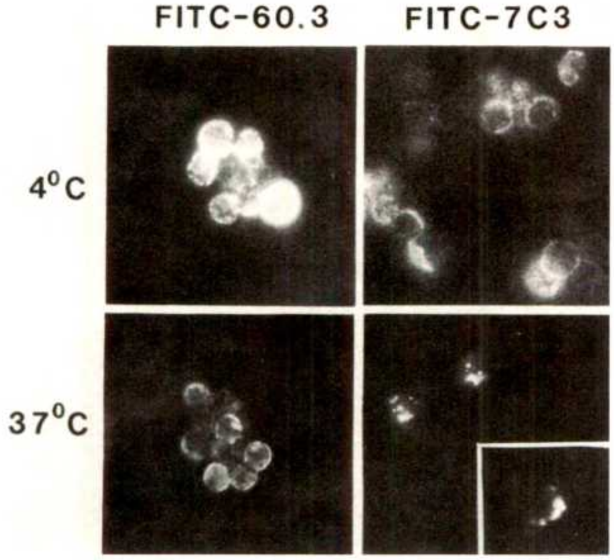

Fig 2. Fluorescence microscopy of FITC-7C3- and FITC-60.3-treated neutrophils. Neutrophils were maintained at 4 °C (upper panels) or 37 °C (lower panels) after incubation with the FITC-linked antibodies (Planapo, ×50). Insert (lower right) demonstrates capping of 7C3 observed under higher magnification (Planapo, ×63).

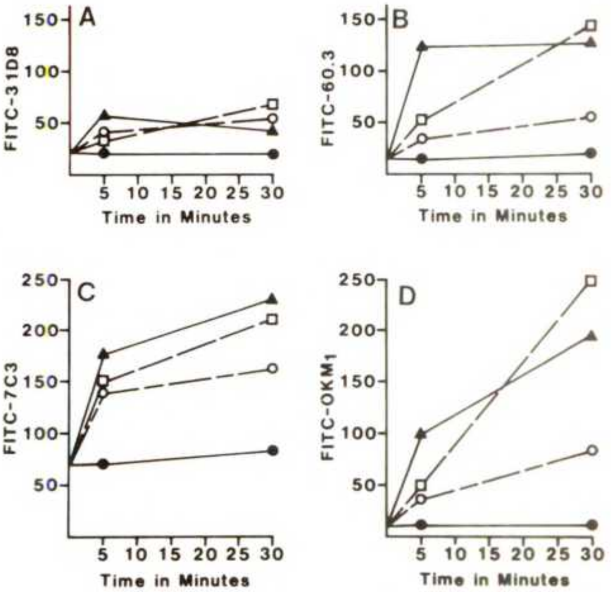

Fig 3. Appearance of new antigen sites on antibody-pretreated PMNs incubated under resting conditions at 4 °C (●—●) or 37 °C (○—○) and stimulated conditions at 37 °C with PMA (▲) or opsonized zymosan (□). Percentage of maximal fluorescence (see Materials and Methods) is shown on the ordinates. Cells were saturated with nonfluorescent antibody at 4 °C and washed free of unbound antibody before incubation and subsequent exposure to FITC-antibody. Data shown are the mean of three experiments.

FITC-OKM1 binding sites by 50% (as assessed by flow cytometry) compared with cells warmed to 37 °C without prior exposure to 7C3. $F(ab')_2$ fragments of 7C3 had an identical effect on PMNs.

Quantitation of antigen binding sites. To determine whether the differential effect of these MAb on PMN function was related to different antigenic density on the cell surface, the number of antigen binding sites for MAb 60.3 and 7C3 was estimated using Scatchard analysis. $F(ab')_2$ fragments of MAb 60.3 detected approximately 123,000 sites per PMN, whereas $F(ab')_2$ fragments of MAb 7C3 detected 156,000 sites per PMN.

Immunoprecipitation. Antigens were immunoprecipitated from cell lysates of ^{125}I-labeled human PMNs using sepharose-linked antibodies (Fig 4). Under reducing conditions using SDS-PAGE, antibody 60.3 identified four bands with approximate molecular weights (mol wt) of 180 kd, 170 kd, 150 kd, and 110 kd. Antibody 7C3 immunoprecipitated three antigens, two of which migrated identically (170 kd, 110 kd) to those identified by antibody 60.3. The third band precipitated by 7C3 migrated with an approximate mol wt of 130 kd. OKM1 detected two bands (approximate mol wt 170 kd and 110 kd) that appeared identical to two of the antigens detected by 60.3 and 7C3.

Although preclearance of the cell lysate with antibody 7C3 neither removed the four bands detected by 60.3 nor the two bands detected by OKM1 (Fig 5), in several experiments the amount of α-chain precipitated by 60.3 and OKM1 was slightly reduced by preclearance with 7C3. Preclearance by 60.3 completely removed the OKM1 antigen and all but the 130-kd band detected by 7C3. Preclearance of the lysate with antibody 7C3 removed all 7C3 antigen, and preclearance with antibody 60.3 removed all of the 60.3 antigen. Preclearance with OKM1 antibody removed the 170-kd and diminished the 110-kd antigens identified by 60.3 but only minimally reduced the 170-kd and 110-kd bands and did not affect the 130-kd band precipitated by 7C3 (data not shown).

DISCUSSION

These studies demonstrate that three MAb, each of which interacts with antigens closely associated with the neutrophil C3bi receptor, have markedly different functional effects on

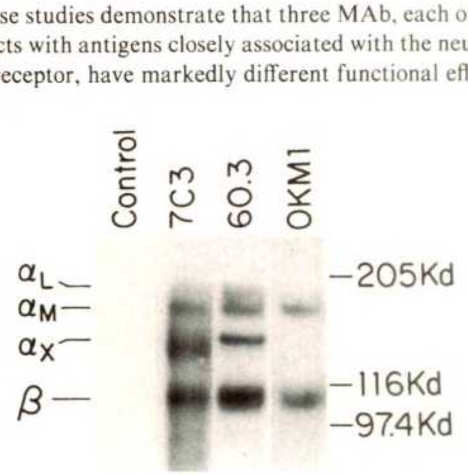

Fig 4. Polyacrylamide gel (7%) electrophoresis of ^{125}I-labeled immunoprecipitates of antibodies 7C3, 60.3, and OKMI from human neutrophils. The immunoprecipitates from the control anti-neutrophil MAb (lane 1), antibody 7C3 (lane 2), antibody 60.3 (lane 3), and antibody OKMI (lane 4) are shown. The α_L, α_M, α_X, and β-subunit designation[17] appears on the left. Mol wt standards are shown on the right. All immunoprecipitates were run under identical reducing conditions.

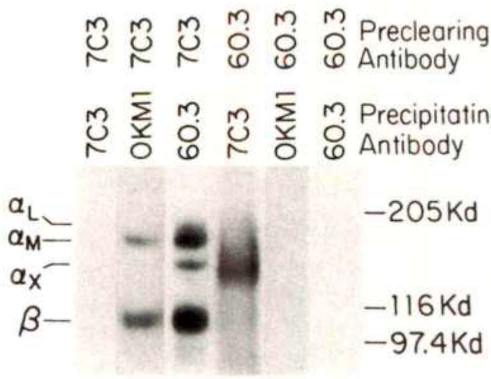

Fig 5. Immunoprecipitation experiments involving the three MAb. ^{125}I-Granulocyte lysates were first immunoprecipitated with the designated "pre-clearing antibody" before immunoprecipitation with the "precipitating antibody." The latter immunoprecipitates were subjected to SDS-7% PAGE. The α_L, α_M, α_X, and β-subunit designation appears on the left. Mol wt standards are shown on the right.

the cell. Antibody 60.3 demonstrated immediate, profound inhibition of the neutrophil oxidative response to C3bi-coated particles that could be reversed after removal of the antibody. Antibody 7C3 demonstrated a time-dependent, largely irreversible inhibition of the neutrophil oxidative response to opsonized zymosan that correlated with capping of the bound antibody. Antibody OKM1 had little effect on PMN respiratory burst activity in response to zymosan. Most striking was the observation that all three antigens recognized by these antibodies demonstrated the capacity for up-regulation after stimulation, although cellular responsiveness was not similarly restored.

The specificity of these antibodies for the active site of the C3bi receptor could be inferred from the results of these studies. Antibody 60.3 had an immediate effect on both particle adherence and O_2^- generation, suggesting that this antibody binds to an epitope close to the active site of the C3bi receptor. Conversely, antibody 7C3 affected these two functions in a time-dependent manner, suggesting that some post-binding step mediated the inhibitory effects of this antibody. The correlation of 7C3's effects with the marked capping of the antibody suggests that the neutrophil may undergo structural alterations after 7C3 binding that, with time, leave the cell less capable of functional responses. Finally, under our conditions, antibody OKM1 seemed to bind an epitope distinct from the active site of the C3bi receptor. Neither O_2^- generation nor particle adherence was markedly inhibited by this antibody, in agreement with previous findings that demonstrated only minimal inhibition of the binding or uptake of EC3bi to PMNs by OKM1.[20]

The functional defects induced by these MAb appeared to be unrelated to the kinetics of antibody binding or the number of antigen binding sites. All three MAb bound in a rapid and saturable manner. Furthermore, the number of antigen binding sites was comparable for MAb 60.3 and 7C3.

Klebanoff et al have reported that the binding of C3bi-coated erythrocytes to PMNs and monocytes is inhibited by treatment of these cells with antibody 60.3.[6,21] Phagocytosis of serum-opsonized zymosan and myeloperoxidase-mediated protein iodination were also inhibited. In the present studies, we have confirmed the ability of antibody 60.3 to inhibit binding of opsonized zymosan by PMNs as assessed both microscopically and by flow cytometry, and by the subsequent triggering of O_2^- release.

It is unlikely that 7C3 binds directly to the active site of the C3bi receptor, since (a) both C3b- and C3bi-coated erythrocytes exhibit decreased binding to 7C3-treated PMNs (54% and 64% of control, respectively[3]) and (b) the inhibitory effects of 7C3 on particle adherence and O_2^- generation were not immediate. Rather, the interference of C3bi-mediated triggering by 7C3 may be due to changes in the distribution of PMN membrane receptors induced by capping of the CR3 complex, an observation first noted by Melnick et al[4] and confirmed here. Of note, the capping of MAb 7C3 and its antigen did not result in co-capping of the OKM1 or 60.3 antigen. The effects of 7C3 on cell attachment and internalization were not as profound as those seen using antibody 60.3 and, consistent with previous studies,[3] suggested a significant postbinding effect in the ability of 7C3 to interfere with O_2^- generation.

The antigens detected by these three antibodies—OKM1, 60.3, and 7C3—appear to belong to a family of structurally related leukocyte surface glycoproteins. Each has a distinct, higher mol wt α-subunit that is noncovalently associated with a common β-subunit.[22] These α-subunits have been designated α_L (a 180-kd glycoprotein detected by the LFA series of MAb[23]), α_M (a 170-kd glycoprotein detected by anti-Mac-1,[24-26] anti-Mol,[27,28] and OKM1[7]), and α_X (a 150-kd glycoprotein reactive with anti-Leu M5 MAb[29]). The variability in the reported mol wt may be related to the degree of glycosylation of the antigens.

Antibodies OKM1,[7] anti-Mac-1,[24-26] and anti-Mol[27,28] all immunoprecipitate the same mol wt heterodimer. Immunoblotting experiments have demonstrated that these antibodies bind to the α_M-subunit and coprecipitate the β-chain because of its noncovalent association with the α_M-chain.[22] Because these three antibodies do not competitively block the binding of one another, they identify distinct epitopes on an identical antigen. This α_M-subunit is present on PMNs, monocytes, and natural killer cells, but not on lymphocytes.[25,26,28] The relative mol wt of the OKM1 antigen reported here (Fig 4) is consistent with that described in previous studies. Although studies to date using both normal cells[20] and cells from patients genetically deficient in this antigen[30] indicate that OKM1 precipitates the C3bi receptor, the data reported here suggest that OKM1 does not bind to the active site of the receptor.

Antibody 60.3 appears to be closely related to MAb TS1/18, which binds to the β-subunit shared by α_L, α_M, and α_X.[22] This presumptive binding site may explain (a) why these two antibodies bind to lymphocytes in addition to PMNs, monocytes, and natural killer cells, and (b) why both antibodies immunoprecipitate all three α-chains and the common β-chain from cell lysates (Fig 4).[5,22,23] Binding to this site is consistent with the ability of antibody 60.3 to remove the OKM1 antigen from PMN cell lysates (Fig 5).

These experiments indicated that the 60.3 antibody coprecipitated the same α_M antigen detected by OKM1.

Antibody 7C3 apparently binds to an antigen associated with this family of leukocyte differentiation antigens, since it immunoprecipitates α_M and β-chains similar to 60.3 and OKM1 (Fig 4). However, the presumptive binding site of 7C3 resides in an antigen of approximate mol wt of 130-kd that is loosely associated with this complex. After preclearing with antibody 7C3, 60.3 immunoprecipitates all four antigens from the cell lysate (Fig 5). However, preclearing with 60.3 removes all 7C3 antigen except the 130-kd moiety (Fig 5). Thus this 130-kd antigen must be loosely associated with the α_M and β-chains. Alternatively, 7C3 could bind to an epitope shared by the three antigens.

Consistent with their shared antigenic similarity, the 60.3, OKM1, and 7C3 antigens are rapidly up-regulated under specific conditions. Although some of the apparent "up-regulation" of antigen undoubtedly represented loss of surface antibody, the degree of up-regulation exceeded that which could be explained by simple dissociation of antibody from cell surface antigens. By indirect immunofluorescence, approximately 50% of 7C3, 35% of OKM1, and 20% of 60.3 was shed during warming at 37 °C over 30 minutes. Such shedding could account for the newly detected sites observed with warming of antibody-saturated cells (Fig 3) but could not account for the extent of up-regulation after membrane stimulation. Under the latter conditions, cells bound 150% to 220% of the amounts of antibody bound to unstimulated cells. These studies did not address whether shed antibody was displaced as antigen–antibody complexes or as antibody alone. It is unlikely that antibodies were internalized by the cell, since experiments using ^{125}I-labeled antibodies paralleled the results with FITC-labeled antibodies.

The factors modulating receptor up-regulation are incompletely defined, although all share membrane perturbation or receptor ligand interaction.[31,32] The α_M antigen (Mol-α antigen) has been identified in a cytoplasmic granular fraction of neutrophils, as well as in the plasma membrane.[33] Degranulation of the cell results in translocation of new antigen to the plasma membrane and a marked increase in the expression of surface antigen.[33] It seems likely that the antigens detected by OKM1, 60.3, and 7C3 undergo similar translocation from cytoplasmic granule stores. Of interest, however, was the finding that the up-regulation of 7C3 antigen was not associated with complete cellular recovery as assessed by subsequent O_2^- generation by opsonized zymosan. As noted above, this may reflect significant postbinding effects on cells preincubated with 7C3.

These experiments demonstrate that MAb binding at similar sites on the neutrophil may have markedly different functional effects. The specific antibody binding site as well as conditions of incubation, temperature, and cell activation all may play significant and distinct roles in determining the final observed functional effect of antibodies on cells. Such conditions should be carefully assessed in future studies of the effects of MAb on cellular physiology.

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Abnormal In Vitro Proliferation and Differentiation of T Colony-Forming Cells in Patients With Lymphadenopathy Syndrome

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Patients with acquired immunodeficiency syndrome (AIDS) present impaired colony growth and in vitro differentiation capacity of peripheral blood and bone marrow T colony-forming cells (T-CFC). We show that peripheral blood, bone marrow, and lymph node T-CFC from patients with persistent lymphadenopathy syndrome (LAS), a syndrome that can precede AIDS, displayed similar abnormalities. Indeed, peripheral blood T-CFC generated a low number of colonies in seven out of 12 patients, and almost no colonies were obtained from bone marrow cells of all patients. The simultaneous study of T-CFC from peripheral blood and lymph node mononuclear cells seems to provide a reliable indicator for the risk of developing AIDS. The six patients who developed AIDS displayed extremely low numbers of peripheral blood T-CFC (13 ± 17 colonies per 5 × 10^4 cells), and in two of them, no colonies could be obtained from lymph node T-CFC. The remaining patients who had not developed AIDS displayed a higher number of peripheral blood T-CFC (141 ± 113 per 5 × 10^4 cells) and lymph node T-CFC, which, in addition, preserved their clonogenic capacity. In some patients, peripheral blood and lymph node, but not bone marrow, T-CFC were capable of generating colonies in the absence of added growth factors or mitogens, whereas in others, colony formation was obtained with purified interleukin 2 (IL 2) alone. Both spontaneous and IL 2-induced colony formation was abrogated by a monoclonal antibody against the IL 2 receptor. Taken together, these findings suggest that at least some T-CFC expressed IL 2 receptors. Colonies generated either in the presence or in the absence of added growth factors were composed of T4$^+$, T6$^+$, and T8$^+$ cells, indicating impaired in vitro T-CFC differentiation. These findings indicate that a dramatic quantitative and qualitative impairment of the proliferation and differentiation of peripheral blood and lymph node T-CFC precedes the clinical evolution from LAS to AIDS.

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A NUMBER OF ASSAYS for the growth of T cell colonies in semisolid media have been developed[1–5] to detect and study the differentiation capacity of peripheral blood and bone marrow T colony-forming cells (T-CFC) in normal subjects[6–8] and in patients with a variety of pathological conditions.[9–11]

In normal subjects, T-CFC have either an immature (E^-, $T3^-$) phenotype (displaying neither receptors for sheep erythrocytes nor T3 molecule) or presumably mature (E^+, $T3^+$) phenotype.[7,8] T cell colony growth from peripheral blood and bone marrow T-CFC requires stimulation with phytohemagglutinin (PHA) or the addition of interleukin 2 (IL 2)-containing conditioned medium to the cultures.[2,7,8] Colonies comprise mature T cells bearing mainly the T3$^+$ and T4$^+$ surface phenotypes,[7,8] but some T3$^+$ and T8$^+$ cells have also been detected.[12]

The lymphadenopathy syndrome (LAS) is defined as a syndrome of unexplained lymphadenopathy of more than three months' duration at two or more extra-inguinal sites.[13–16] Many patients manifest nonspecific symptoms (weight loss, oral thrush, diarrhea, unexplained fever), and prospective epidemiological studies[17] suggest that about 10% of patients will ultimately develop either opportunistic infections or Kaposi's sarcoma, which characterizes the acquired immunodeficiency syndrome (AIDS).[18] Moreover, LAS and AIDS patients present low number of T lymphocytes, especially the T4$^+$ subset, and qualitative abnormalities of the immune system (decreased proliferative responses to mitogens, allo- and auto-antigens, lack of T cell help for immunoglobulin synthesis, and decreased production of IL 2 by lymphocytes).[13–16,19,20] PHA-induced colony formation from peripheral blood T-CFC from LAS and AIDS patients[21] and in hemophiliacs with lymphadenopathy[22] showed impaired colony growth capacity, which could be restored with exogenous IL 2. We have recently reported that the colony-forming capacity of peripheral blood and bone marrow T-CFC from AIDS patients was significantly impaired.[23] Moreover, the patients' T-CFC displayed extremely low self-renewal capacity. Interestingly, T cell colonies were composed, unlike normal colonies, of relatively immature cells bearing the T6$^+$, T4$^+$, T8$^+$.[23] In the present study, we raised the question whether peripheral blood, bone marrow, and lymph node T-CFC of LAS patients show similar abnormal in vitro proliferation and differentiation capacities. The results indicate that patients' peripheral blood and bone marrow T-CFC displayed impaired proliferative capacities, which were more pronounced in patients who subsequently developed AIDS. Conversely, lymph node T-CFC of LAS patients who did not develop AIDS were more clonogenic than the T-CFC of patients who progressed to AIDS. In addition, peripheral blood and lymph node T-CFC showed impaired in vitro differentiation, similar to that observed in AIDS patients.[23]

MATERIALS AND METHODS

Patients. Heparinized peripheral blood, bone marrow, or lymph node biopsy specimens were obtained from 19 consecutive patients

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with LAS. All but two patients, a Haitian woman (No. 16) and an intravenous drug user (No. 4), were male homosexuals All patients fulfilled the LAS criteria.[16] One patient (No. 15) presented with general symptoms, two (No. 2 and No. 17) had syphilis, and one (No. 14) had zoster (Table 1). Cytologic studies of lymph node biopsy sections revealed no evidence of malignant disease and were compatible with the diagnosis of LAS.[24] Six patients (14 through 19) developed AIDS within a year (three to 12 months) after T-CFC investigation. All patients were studied on presentation, before any treatment was applied, and displayed serum antibodies against the lymphadenopathy-associated virus (LAV) as detected by enzyme-linked immunosorbent assay[17] and Western blot.[25] Seven clinically healthy male homosexuals and 17 healthy heterosexuals were also studied as controls. All healthy male homosexuals but one, as well as all healthy heterosexuals, were seronegative for LAV antibodies.[23] Informed consent for these studies was obtained from all patients.

Cell separation. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were obtained by Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density-gradient centrifugation ($d = 1.077$ g/cm^3). Interface cells were washed twice with Hanks' balanced salt solution (HBSS; Inst Pasteur Production, Marnes-la-Coquette, France) and resuspended (10^6 cells per milliliter) in growth medium (α-modified Eagle's medium [α-MEM]; GIBCO, Grand Island, NY). Lymph node biopsy specimens were mechanically dissociated, cells were suspended in HBSS, and lymph node mononuclear cells (LNMCs) were obtained by density centrifugation as described earlier. Their viability, tested by trypan blue dye exclusion, was always >90%.

In some experiments, cells were further separated on the basis of sheep red blood cell (SRBC)-rosette formation with AET (2-amino ethylisothiouronium hydrobromide; Sigma Chemical Co, St Louis)-treated SRBCs[26] and a second density-gradient centrifugation Interface cells were thereafter treated with OKT3 monoclonal antibody (MoAb) (Ortho, Raritan, NJ) (50 μL/10^6 cells in a final dilution 1:400 vol/vol, at 4 °C) and pretested low Tox-H rabbit complement (Cedarlane, Ontario; 70 to 90 μL/10^6 cells in a final dilution 1·8 vol/vol) at 37 °C. This cell fraction contained less than 2% E$^+$ and no OKT3$^+$ cells and will be referred to as E$^-$OKT3$^-$ cells. E$^+$ cells were obtained from the pellet of the second density centrifugation after hypotonic lysis of the SRBCs. Their viability, tested by trypan blue dye exclusion, was >90%.

T cell colony assay. Mononuclear cells (5×10^5 cells per milliliter) or cell fractions were seeded in 0.8% methylcellulose (Fluka, Bachts, Switzerland) in α-MEM supplemented with 20% (vol/vol) heat-inactivated fetal calf serum (FCS; GIBCO), 2 mmol/L-glutamine and antibiotics, in the presence and the absence of 20% (vol/vol) PHA-leukocyte conditioned medium. This conditioned medium (PHA-LCM) was a seven-day supernatant from PHA (PHA-M 1%, vol/vol, DIFCO Lab, Detroit)-stimulated normal PBMCs (10^6 cells per milliliter in α-MEM supplemented with 10% FCS and 2 mmol/L L-glutamine) and contained 0.2 U/mL of IL 2 (1 U = 50 BRMP U) activity when tested on an IL 2-dependent human T cell line.[27] In addition, cells were seeded in the presence of 0.3 U/mL of PHA-free semipurified IL 2. This was obtained from 48-hour supernatants of PHA-M (1%, vol/vol)–stimulated normal PBMCs, precipitated with ammonium sulfate, filtered on Sephadex G 75 superfine columns (Pharmacia), and represented the pooled 15-kd to 22-kd fractions that contained the IL 2 activity (5 U/mL). PHA-free IL 2 could not induce cell proliferation or colony formation from normal PBMCs in the absence of PHA (kindly provided by Dr F. Triebel, UER Pitie Salpetriere, Paris).[8,28] One tenth milliliter of the methylcellulose-containing cell preparation was seeded per well in 96-well flat-bottomed microtest plates (Nunc, Roskilde, Denmark). Cultures were incubated for five to seven days at 37 °C in 5% CO^2 in air, and aggregates containing more than 50 cells were counted as colonies under an inverted microscope.

In some experiments, 5×10^5 cells per milliliter were incubated for 45 minutes at 37 °C with increasing concentrations (10^{-3} to 10^{-7}; vol/vol) of anti-Tac monoclonal antibody, which recognizes the IL 2 receptor (IL 2-R; anti-Tac kindly provided by Dr TA Waldmann,

Table 1. Hematologic Characteristics of LAS Patients

		Positive Cells (%)										
		Peripheral Blood			Bone Marrow			Lymph Node				
Patient No.	Diagnosis	ALC (per μL)	T4	T8	T11	DR	Tac	T11	T4	T8	Tac	DR
1	LAS	2,200	43	37	3	0	2	7	0	9	3	31
2	LAS	2,080	27	25	2	0	2	ND	ND	ND	ND	ND
3	LAS	1,100	24	63	22	15	7	ND	ND	ND	ND	ND
4	LAS	1,989	18	71	32	5	0	23	8	9	0	44
5	LAS	2,795	31	47	13	13	0	42	23	18	28	60
6	LAS	1,176	27	40	ND	ND	ND	8	2	7	0	28
7	LAS	1,200	27	47	ND	ND	ND	17	16	12	1	27
8	LAS	1,508	18	55	ND	ND	ND	21	12	2	0	69
9	LAS	1,300	21	39	ND	ND	ND	26	8	23	23	44
10	LAS	1,710	26	32	ND	ND	ND	ND	ND	ND	ND	ND
11	LAS	1,428	27	65	ND	ND	ND	ND	ND	ND	ND	ND
12	LAS	1,390	22	56	ND	ND	ND	ND	ND	ND	ND	ND
13	LAS(KS)	1,966	29	48	ND	ND	ND	ND	ND	ND	ND	ND
14*	LAS-AIDS(KS)	1,300	12	57	4	0	0	ND	ND	ND	ND	ND
15*	LAS-AIDS(KS)	1,889	15	62	ND	26	4	32	20	28	4	19
16*	LAS-AIDS(KS)	1,289	1	47	10	0	9	ND	ND	ND	ND	ND
17*	LAS-AIDS(KS + OI)	1,920	13	73	9	2	1	ND	ND	ND	ND	ND
18*	LAS-AIDS(KS)	1,200	18	36	ND	ND	ND	29	21	17	3	39
19*	LAS-AIDS(KS + OI)	1,180	23	36	ND	ND	ND	38	25	27	3	47

ALC, absolute lymphocyte counts; ND, not done; KS, Kaposi's sarcoma; OI, opportunistic infections.

*Patients who subsequently developed AIDS.

National Institutes of Health).[29] The cells were washed extensively and seeded in methylcellulose in the absence or presence of IL 2 as described.

Clonogenic capacity of T-CFC. The clonogenic capacity of T-CFC from PBMCs and LNMCs was determined by incubating 10^6 cells per milliliter in growth medium supplemented with 10% FCS and 2 mmol/L L-glutamine for three to five days. Extensively washed cells were seeded in methylcellulose (5×10^5 viable cells per milliliter), in either the presence or the absence of added growth factors as described ("delayed plating").[30]

Phenotypic characterization of PBMCs, BMMCs, LNMCs, and colony cells PBMCs, BMMCs, LNMCs were phenotyped using a panel of monoclonal antibodies, including the OKT series (Ortho); the T11 (Coultronics, Margency, France), which recognizes SRBC receptors; and an anti-HLA–class II (BM-50; kindly provided by Dr D. Charron, UER Pitie Salpetriere, Paris). Positive cells were counted with an Ortho Spectrum III Analyser, using a minimum sample size of 2,000 cells.

Colonies of the same size and morphology were individually picked, pooled, dissociated, and washed with HBSS. Colony cells were resuspended in phosphate-buffered saline supplemented with 0.01% sodium azide and 2% FCS, and phenotyped by indirect immunofluorescence using the OKT series monoclonal antibodies Nonspecific immunofluorescence was always <5%. At least 50 to 100 cells were evaluated for each determination

Statistical analysis Statistical comparison of the mean values was performed using the Student's *t* test.

RESULTS

PBMC, BMMC, and LNMC phenotypes. As expected, $T4^+$ cells were decreased (range, 1% to 43%), while $T8^+$ cells ranged from 25% to 73% (Table 1). All patients displayed, to varying degrees, a low $T4^+$:$T8^+$ ratio (range, 0.02 to 1.16; normal values, 1.5 to 2.3). Patients who subsequently developed AIDS (14 through 19) had a significantly lower $T4^+$:$T8^+$ ratio than the remaining patients (mean ± SD:0.27 ± 0.21 *v* 0.23 ± 0.17, respectively; $P < .05$). In these patients, both the proportion and the absolute number of $T4^+$ (13.7% ± 7.4% and 198 ± 102 cells per cm^3, respectively) were lower than in those who did not develop AIDS (26.1% ± 6.5% and 483 ± 226 cells per cm^3, respectively). Tac^+ cells were always <5%.

Phenotypic characterization of BMMCs showed no significant peripheral blood contamination as evaluated by the proportion of $T3^+$ and $T11^+$ cells, except in two patients (No. 3 and No. 4, respectively; Table 1). No $T6^+$ cells were found in any patient, whereas Tac^+ cells were ranged between 0% and 9% (Table 1).

Phenotypic characterization of LNMCs showed 9% to 46% $T3^+$ cells (mean ± SD:32.3% ± 12%; Table 1) and in four of them (1, 4, 6, 9), $T4^+$ cells were less than 10%. $T8^+$ cells were usually <30%, and the $T4^+$:$T8^+$ ratio ranged from 0 to 1.2. In two patients (No. 5 and No. 9; Table 1), 23% and 28% of the LNMCs were Tac^+, respectively.

T cell colony formation. PBMCs from seven out of 12 patients generated low numbers of colonies (less than 50 colonies/5×10^4 cells). The colony formation from patients who did not develop AIDS was lower than in normal heterosexuals (mean ± SD, 141 ± 113 and 370 ± 179 colonies/5×10^4 cells, respectively; $P < .001$). Patients who subsequently developed AIDS generated a dramatically decreased number of colonies (13 ± 17 colonies/5×10^4 cells) (Table 2). There was no correlation between the absolute number of $T4^+$ cells and the number of colonies. Furthermore, T cell colony growth from seven clinically healthy male homosexuals who were at risk of developing AIDS (described in detail elsewhere[23]) was also significantly decreased (208 ± 46 colonies/5×10^4 cells; $P < .01$).

Bone marrow T-CFC could not generate colonies, irrespective of the subsequent development of AIDS. Conversely, in six out of eight patients who did not develop AIDS, lymph node T-CFC yielded a relatively high number of colonies (more than 100 colonies/5×10^4 cells). This contrasts with the absence of colony growth from LNMCs of patients No. 18 and No. 19 and the 24 colonies obtained from patient No. 14 (Table 2), who subsequently developed AIDS.

To determine more precisely the clonogenic capacity of presumably "mature" and immature T-CFC,[8] fractionated E^+ and E^- $T3^-$ PBMCs and LNMCs were also tested for T cell colony growth. As shown in Table 3, peripheral blood E^+ and E^- $T3^-$ cells (except in one patient) were unable to generate colonies, although colony growth could be obtained from unfractionated PBMCs, suggesting that cellular cooperation(s) are important for T cell colony growth. Lymph node E^+ cells from two patients (No. 8 and No. 18; Table 3)

Table 2. Induced T Cell Colony Growth in LAS Patients

Patient No	Peripheral Blood	Bone Marrow	Lymph Node
1	285 ± 24	0	290 ± 31
2	300 ± 22	0	ND
3	36 ± 7	0	ND
4	40 ± 3	24 ± 2	130 ± 12
5	8 ± 1	0	120 ± 8
6	ND	ND	216 ± 18
7	ND	ND	120 ± 10
8	ND	ND	216 ± 18
9	ND	ND	82 ± 3
10	ND	ND	116 ± 7
11	128 ± 5	ND	0
12	209 ± 12	ND	ND
13	120 ± 8	ND	ND
14*	10 ± 2	4 ± 1	24 ± 3
15*	32 ± 4	0	ND
16*	8 ± 1	0	ND
17*	0	ND	ND
18*	ND	ND	0
19*	ND	ND	0
Healthy Homosexuals (n = 7)	203 ± 46	ND	ND
Healthy Heterosexuals (n = 17)	370 ± 179	246 ± 86	ND

Cells (5×10^5/mL) were seeded in methylcellulose in the presence of PHA-LCM as described in Materials and Methods Aggregates containing more than 50 cells were counted as colonies. Results are expressed as the mean number of colonies ± SD/5×10^4 seeded cells from triplicate cultures. ND, not done.

*Patients who subsequently developed AIDS.

Table 3. Colony Formation From Different Cell Fractions of LAS Patients' PBMCs and LNMCs

Patient No	Source of Cells	Unfractionated MNC	E$^+$ Cells	E$^-$OKT3$^-$ Cells
1	PBMC	285 ± 24	0	0
2	PBMC	300 ± 22	0	287 ± 17
3	PBMC	36 ± 7	0	0
14*	PBMC	10 ± 2	0	0
16*	PBMC	8 ± 1	0	0
6	LNMC	216 ± 19	392 ± 28	ND
8	LNMC	82 ± 12	0	0
10	LNMC	0	0	0
18*	LNMC	0	330 ± 23	0
19*	LNMC	0	0	0

Unfractionated MNC, E$^+$, and E OKT$_3$/ (5 × 10^6/mL) were seeded in methylcellulose in the presence of PHA-LCM, as described in Materials and Methods. Aggregates containing more than 50 cells were counted as colonies. Results as expressed as mean number of colonies ± SD/5 × 10^4 seeded cells from triplicate cultures. ND, not done.

*Patients who subsequently developed AIDS.

generated a high number of colonies, but E-T3$^-$ cells did not. It is noteworthy that in patient No. 18, who subsequently developed AIDS, unfractionated LNMCs did not generate colonies, whereas E$^+$ cells did.

Colony formation in the absence of added growth factors and in the presence of purified IL 2. Both unfractionated PBMCs (in four out of 11 patients) and LNMCs (in four out of 11 patients) generated colonies in the absence of added growth factors or mitogenic stimulation (Table 4). To determine whether IL 2-R were involved in this spontaneous colony growth, cultures were performed after incubating cells with anti-Tac MoAb. As shown in Fig 1, spontaneous colony formation could be abrogated with anti-Tac MoAb, irrespective of the source of the cells. Low amounts (10^6 vol/vol) of anti-Tac inhibited the colony formation in all cases.

Table 4. Spontaneous and IL 2-Induced T Cell Colony Formation

Patient No.	Source of Cells	Spontaneous	IL 2
1	PBMC	64 ± 9	503 ± 37
2	PBMC	42 ± 6	156 ± 12
3	PBMC	0	4 ± 1
5	PBMC	4 ± 2	4 ± 3
11	PBMC	0	0
12	PBMC	142 ± 12	0
13	PBMC	0	0
14*	PBMC	0	0
15*	PBMC	82 ± 9	0
16*	PBMC	0	0
17*	PBMC	0	0
1	LNMC	278 ± 19	160 ± 13
4	LNMC	122 ± 17	26 ± 8
5	LNMC	320 ± 21	20 ± 4
6	LNMC	0	12 ± 2
7	LNMC	26 ± 3	146 ± 17
8	LNMC	0	31 ± 5
9	LNMC	0	20 ± 3
10	LNMC	0	0
14*	LNMC	0	0
18*	LNMC	0	0
19*	LNMC	0	0

PBMCs and LNMCs (5 × 10^5/mL) were seeded in methylcellulose in the absence or presence of added IL 2 without mitogenic stimulation and incubated as described in Materials and Methods. Results are expressed as mean ± SD of colonies per 5 × 10^4 seeded cells from triplicate cultures.

*Patients who subsequently developed AIDS.

Biochemically purified IL 2 induced colony growth from PBMCs and LNMCs in two and three patients (1 and 2 and 7 through 9; Table 4), respectively, without previous mitogenic stimulation. Similar results were obtained when, in some experiments, genetically recombinant (Biogen, Geneva, Switzerland) IL 2 was substituted for biochemically semipurified IL 2 (not shown). Because the induction of colony formation with purified IL 2 seems to indicate that some T-CFC could express IL 2-R,[31] both PBMCs and LNMCs were incubated with anti-Tac MoAb before seeding in the presence of IL 2. As shown in Fig 2, anti-Tac abrogated the IL 2-induced colony formation. Conversely, a pool of three anti-HLA–Class II MoAbs, used as control, could not modify the plating efficiency. It is worthy to note that semi-purified IL 2 inhibited T cell colony growth from PBMCs of two patients (No. 12 and No. 15; Table 4) and from LNMCs of three patients (1, 4, and 5; Table 4). Interestingly, BMMCs from seven patients (1 through 5 and 14 through 16) did not generate either spontaneous or IL 2-induced colonies, although in two of them (No. 3 and No. 16; Table 1) 7% and 9% of the cells, respectively, were Tac$^+$.

Phenotypic characterization of colonies. Colonies generated from PBMCs and LNMCs in the presence of PHA-

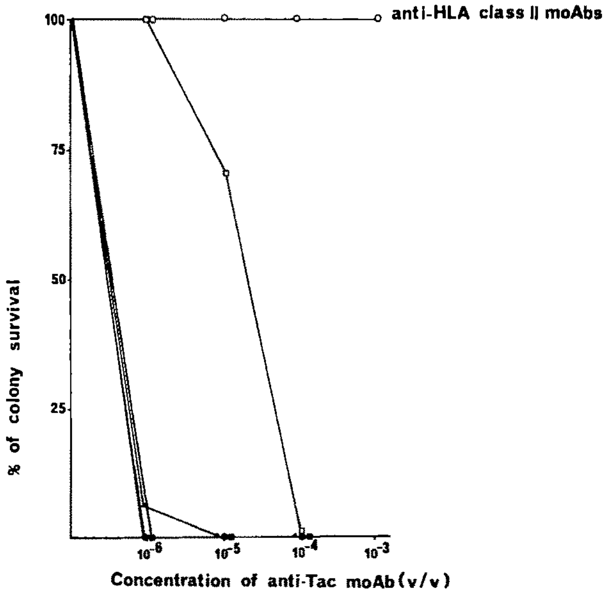

Fig 1. Inhibition of IL 2-induced T cell colony formation with anti-Tac MoAb. PBMCs or LNMCs (5 × 10^5 cells per milliliter) were incubated with increasing concentrations of either anti-Tac or a pool of three anti-HLA class II MoAbs at 37 °C for one hour. Extensively washed cells were seeded in methylcellulose in the presence of purified IL 2 as described in Materials and Methods. Each sign represents a different patient: ■—■, No. 1 (PBMC); ▲—▲, No. 2 (PBMC); □—□, No. 5 (PBMC); ●—●, No. 4 (LNMC).

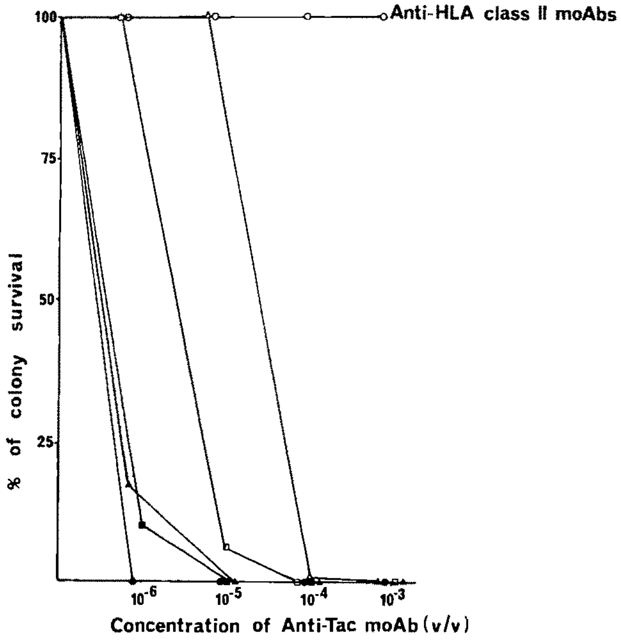

Fig 2. Inhibition of spontaneous T cell colony formation with anti-Tac MoAb. PBMCs or LNMCs (5×10^5 cells per milliliter) were incubated with increasing concentrations of either anti-Tac or a pool of three anti-HLA class II MoAbs at 37 °C for one hour. Extensively washed cells were seeded in methylcellulose in the absence of added growth factors and mitogenic stimulation as described in Materials and Methods. Each sign represents a different patient: ●—●, No. 1 (PBMC); ▲—▲, No. 2 (PBMC); △—△, No. 5 (LNMC); ■—■, No. 15 (PBMC); □—□, No. 4 (LNMC).

LCM were composed of lymphoblastoid cells bearing T cell surface markers (Table 5). Forty-eight percent to 83% of the colony cells were T3$^+$, whereas 46% to 94% were T4$^+$ and 58% to 95% were T8$^+$. In all but one case (No. 5; Table 5), more than 50% of the colony cells were T6$^+$, unlike T cell colonies from healthy heterosexuals. Moreover, induced colonies from clinically healthy male homosexuals contained a significantly higher proportion of T6$^+$ cells than colonies from normal heterosexuals ($P < .001$; Table 5).[23]

Spontaneous colonies from PBMCs and LNMCs were smaller than induced colonies (up to 100 cells per colony), and were composed of lymphoblastoid cells bearing T cell surface markers (Table 5). The phenotype of cells from spontaneous colonies was not significantly different from that of induced colonies.

Clonogenic capacity of T-CFC. The proliferative capacity of peripheral blood, bone marrow, or lymph node T-CFC was also tested after incubating the cells in liquid culture for three to five days in the absence of added growth factors or mitogens before seeding in methyl cellulose. This "delayed" plating has been proposed as a measure of the proliferative capacity of the clonogenic cells.[30] As shown in Table 6, "delayed" plating did not, in general, enhance the spontaneous plating efficiency and in two of three patients (No. 1 and No. 2; Table 6), completely abrogated the induced colony growth. However, delayed plating of LNMCs from patient No. 18, who subsequently developed AIDS, significantly enhanced the induced colony formation (Table 6). Colonies obtained by "delayed" plating were also of T cell origin, since they were counted of more than 75% T3$^+$ cells.

DISCUSSION

The results reported here indicate that LAS patients present important quantitative and qualitative abnormalities of peripheral blood and bone marrow T-CFC. The bone marrow T-CFC from all patients failed to generate colonies and the peripheral blood T-CFC of several patients, especially those who subsequently developed AIDS, gave rise to a particularly low number of colonies. Similarly, other investigators showed impaired T colony formation from PBMCs of hemophiliacs with lymphadenopathy[22] and AIDS and of LAS patients.[21] However, in these studies,[21,22] T cell colony formation was obtained by PHA stimulation of patients' PBMCs without the addition of IL 2; exogenous IL 2 enhanced the colony growth, suggesting that the observed low plating efficiency could be due to the impaired capacity of patients' T cells to produce IL 2.[20]

In our experiments, colony formation was induced with crude conditioned medium containing both IL 2 and low amounts of PHA (0.001%). Contaminant PHA could not yield colony growth, whereas as little as 0.1 U/mL of IL 2 is sufficient for normal T cell colony formation in the presence of PHA.[8] Moreover, aggregates containing more than 50 cells were considered as colonies, whereas in the abovementioned studies,[21,22] aggregates with as few as 25 cells

Table 5. Phenotypic Characterization of Induced and Spontaneous T Cell Colonies From PBMCs, BMMCs, and LNMCs of LAS Patients

Patient No	Source of Cells	Induced Colonies (%)				Spontaneous Colonies (%)			
		T3	T4	T6	T8	T3	T4	T6	T8
1	PBMC	48	46	56	60	42	40	38	41
2	PBMC	81	88	92	94	80	82	84	87
4	LNMC	83	79	65	83	100	88	92	94
5	LNMC	69	87	34	58	62	50	41	54
Healthy homosexuals (n = 3)	PBMC	79 ± 9	60 ± 11*	50 ± 14*	48 ± 15*	NCG			
Healthy heterosexuals (n = 7)	PBMC	75 ± 5	47 ± 13	17 ± 5	35 ± 4	NCG			

Individually picked colonies were pooled, washed, and dissociated, and colony cells were stained by indirect immunofluorescence with the corresponding MoAb. NCG, no colony growth

$^*P < .01$ (Student's *t* test).

Table 6. Clonogenic Capacity of LAS Patients' T-CFC

Patient No	Source of Cells	Immediate Plating		Delayed Plating•	
		Spontaneous	Induced	Spontaneous	Induced
1	PBMC	64 ± 9	285 ± 24[b]	0	0
2	PBMC	42 ± 6	300 ± 22	0	10 ± 3
14•	PBMC	0	10 ± 2	0	20 ± 4
	PBMC	0	4 ± 1	0	14 ± 2
4	LNMC	122 ± 17	130 ± 12	150 ± 12	242 ± 23
18•	LNMC	0	0	0	236 ± 26

•Cells (5×10^5/mL) were seeded in methylcellulose after 72 hours of cell incubation in α-MEM in the absence of added growth factors or mitogens. Results are expressed as mean number of colonies per 5×10^4 seeded cells from triplicate culture.

•Patients who subsequently developed AIDS

counted as colonies. It is likely that the two culture conditions allow the growth of distinct T-CFC subsets and could explain our observation that IL 2-containing conditioned medium did not enhance the plating efficiency.

Lymph node T-CFC gave rise to a high number of colonies in seven out of eight LAS patients who had not progressed to AIDS and a low number in three patients who subsequently developed AIDS. This observation could be of prognostic importance, since all patients who developed AIDS also yielded an extremely low T cell colony formation from peripheral blood cells. This group of patients could represent an earlier stage during the evolution of the disease, in which T cell precursors are still present in lymph nodes but not in peripheral blood.

In AIDS patients the absolute number of peripheral blood T-CFC was significantly correlated with that of $T4^+$ cells.[23] In the present study no such correlation could be established, suggesting that the low colony formation of peripheral blood T-CFC in LAS patients could precede the $T4^+$ lymphopenia. Moreover, although LAS patients who developed AIDS displayed significantly lower $T4^+$ cells than those who did not develop the full-blown disease, there was an important overlap of the $T4^+$ cell values, making any close correlation between this parameter and the outcome of the patients difficult. Therefore, it seems that the combination of low colony growth of both peripheral blood and lymph node T-CFC of LAS patients has a higher prognostic value than the absolute number of $T4^+$ cells. Recent studies also point out that other immunologic parameters, such as the 2-microglobulin,[31] mitogen-induced lymphocyte stimulation,[32] acid labile interferon,[33] could also be of prognostic value for the evolution of AIDS. It may be essential to determine whether early treatment of these particular patients could prevent or delay the development of AIDS.

In most patients, neither purified cell fractions, known to be capable of generating T cell colonies,[7,8] nor "delayed" plating, considered to be a method of measuring the clonogenic capacity of hematopoietic precursors,[30] could enhance the colony growth. However, in one patient (No. 18) who subsequently developed AIDS, purified lymph node E^+ cells but not unfractionated LNMCs generated colonies (Table 3). These observations suggest that, at least in this patient, inhibitory mechanism(s) could account for the low plating efficiency from LNMCs. Moreover, in patient No. 4, "delayed" plating of LNMCs revealed that T-CFC also displayed a high clonogenic capacity.

The fact that, in some patients, colony growth could be obtained either spontaneously or with biochemically purified and recombinant IL 2, whereas the anti-Tac MoAb abrogated colony formation, indicates that some T-CFC (both lymph node and peripheral blood) are already activated cells bearing IL 2-R.[34,35] The detected low proportion of Tac^+ cells does not contradict this finding, since T-CFC represent a small subset of T cells (usually two to three clonogenic cells per 10^3 PBMCs[8]). A similar phenomenon has been observed in some human malignant T cell lines[36] and in T-CFC from some patients with T cell malignancies.[9] Conversely, the fact that BMMCs in some cases did not generate IL 2-induced colonies although they expressed IL 2-R, suggests that IL 2-R were either not functional or displayed a low affinity for IL 2.[34] Thus the presence of activated T-CFC can probably explain the T cell colony formation in the absence of added growth factors.

In some patients, both biochemically purified and recombinant (not shown) IL 2 inhibited spontaneous colony formation. Although the reasons for this phenomenon are not yet known, we cannot exclude the possibility that IL 2 stimulated suppressor T cells to produce inhibitory activity(ies) for the proliferation of T-CFC.

Unlike colonies from healthy heterosexuals, colonies generated from LAS patients comprised mainly immature cells, bearing the $T4^+$, $T6^+$, $T8^+$ phenotype. The possibility that some colonies comprised mature ($T3^+$, $T4^+$, $T6^-$ or $T3^+$, $T8^+$, $T6^-$) cells cannot be excluded, since pooled colonies were used for phenotypic studies. However, the phenotype of spontaneous and induced colony cells was similar, indicating that the abnormal in vitro differentiation of peripheral blood, bone marrow, or lymph node T-CFC is growth factor independent.

In conclusion, our data indicate that T cell lymphopenia observed in LAS as well as in AIDS patients[23] and in some male homosexuals could be the result of several mechanisms, such as impaired proliferative capacity and decreased number and abnormal in vitro differentiation of T-CFC. In addition, the in vitro study of T-CFC could identify some LAS patients presenting a low number of peripheral blood and lymph node T-CFC, whose risk of developing AIDS is extremely high. Longitudinal studies are in progress in order to verify the prognostic value of this observation.

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T3 Surface Molecules on Adult T Cell Leukemia Cells Are Modulated In Vivo

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Cells from eight patients with adult T cell leukemia (ATL) and from four patients with non-ATL were examined to see if the T3 antigen of these cells could be modulated in vitro. We found a low density of T3 antigen and the presence of Tac antigen on cells from all patients with ATL. The density of T3 antigen on non-ATL cells was normal, and Tac antigen was not detected. Modulation of T3 antigen and an increase in Tac antigen-positive cells occurred when cells from patients with T4 non-ATL were cultured with OKT3 monoclonal antibody (mAb). Those changes in T3 antigen density and the appearance of Tac antigen-bearing cells by OKT3 mAb were not so marked when ATL cells were used. But the modulation of T3 antigen and the increase in Tac antigen-bearing cells by OKT3 mAb were closely related in cells from six ATL patients. These findings suggest that T3 T cell antigen receptor complexes on ATL cells are not functionally "frozen" by leukemic changes and might be modulated in vivo. In addition, modulation of T3 surface antigen on ATL cells was not induced by cultivation with human T cell leukemia virus type I particles and envelope proteins obtained by gene technology.

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ADULT T CELL LEUKEMIA (ATL) has been established as a distinct clinical entity associated with retrovirus infection.[1-4] The surface phenotype of ATL cells characterized by monoclonal antibodies (mAbs) is $T3^+T4^+$-$T8^-T11^+Tac^+$.[5] With improved biochemical characterization of antigens, the functions of antigens recognized by some mAbs have been defined. Tac antigen is known to recognize interleukin 2 (IL 2) receptors or closely associated molecules.[6,7] Reinherz et al[8-10] found that T3 antigen is associated with the T cell antigen receptor (Ti) because T3 mAb coimmunoprecipitated Ti antigen from alloantigen-specific cloned T cells; both T3 and Ti antigens were comodulated by OKT3, anti-Ti mAb, or alloantigens. Our recent analyses of ATL cells using a laser flow cytometry showed that the mean fluorescence intensity (MFI) of T3 antigen significantly decreased on ATL cells, although those of T4 and T11 were normal.[11] The low density of T3 antigen and the presence of Tac antigen on ATL cells are equivalent to the surface phenotype of normal T cells whose T3 antigen is modulated by OKT3 mAb. It is interesting to know whether T3 antigen on ATL cells can still be modulated in vitro or is "frozen" at a low level. If the former is the case, Tac antigen expression on ATL cells might be the secondary phenomenon of T3 antigen modulation. So we studied the modulation of T3 antigen and the induction of Tac antigen by OKT3 mAb simultaneously, using ATL cells from eight patients and malignant T cells from four non-ATL patients.

We also studied the effects of human T cell leukemia virus type 1 (HTLV-I) and envelope gene products of HTLV-I on T3 and Tac antigen expression of ATL cells in order to test whether low density of T3 antigen on ATL cells may be secondary phenomena of chronic stimulation of Ti molecules on ATL cells because these two molecules are noncovalently linked.[8-10]

MATERIALS AND METHODS

Reagents Fluorescein isothiocyanate (FITC)-conjugated OKT3, T4, and T8 mAbs and unlabeled OKT3, T4, and T8 mAbs were purchased from the Ortho Pharmaceutical Co (Raritan, NJ). FITC-conjugated anti-Tac mAb was generated as described elsewhere.[12] FITC-labeled goat $(Fab')_2$ antimouse $(Fab')_2$ fragments of immunoglobulin G (IgG) was from Cappel Laboratories (Cochranville, Pa). Affinity-purified FITC-labeled goat $(Fab')_2$ antihuman IgG was from Tago Inc (Burlingame, Calif). Concanavalin A (Con A) was from Pharmacia (Uppsala, Sweden) and phorbol 12-myristate 13-acetate (PMA) was from Consolidated Midland (Brewster, NY).

Cell culture and cell lines All cell cultures were done in complete medium, RPMI 1640 (Nissui, Tokyo) containing 10% fetal bovine serum (GIBCO, Grand Island, NY), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mmol/L L-glutamine. Cell counts and viabilities were determined visually with trypan blue. The MT-2 cell line cells[13] were kindly provided from Dr I. Miyoshi (Kochi Medical University, Kochi, Japan). HUT 102 cell line cells[4] were from Dr R.C. Gallo (National Cancer Institute, Bethesda, Md). Peripheral blood mononuclear cells from patients and ten healthy donors (aged 26 through 38) were separated from heparinized blood by Ficoll-Conray density-gradient centrifugation. Normal T cells were purified by sheep red blood cell rosette-forming methods. After being washed three times with phosphate-buffered saline (PBS), cells were cultured with or without various reagents in a humid atmosphere with 5% CO_2 at 37 °C. For short-term culture, recombinant interleukin 2 (IL 2), which was kindly provided by Dr J. Hamuro (Ajinomoto Pharmaceutical, Yokohama, Japan), was used. Cells were fed with IL 2 (1 U/mL) every two days.

Patients Diagnoses of patients were based on accepted criteria for clinical, hematologic, and laboratory findings. Antibody to ATL virus-associated antigens (anti–ATLA-Ab) in the sera was measured by indirect immunofluorescence (IF) as described by Hinuma et al,[4] using MT-1 cells Antibodies to virus-associated cell surface membrane antigens (anti–MA-Ab) were also measured, using HUT 102 cells as described by Robert-Guroff et al.[14] Briefly 50 μL of 1:100 diluted patient's sera was added to 10^6 cells and incubated for 30 minutes on ice. After washing twice with PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (NaN_3), 50 μL

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of FITC-conjugated affinity-purified antihuman IgG (1.40 dilution) was added. Cells were washed twice after another 30 minutes of incubation on ice and analyzed by a laser flow cytometry system (Spectrum III/Ortho Diagnostic Systems, Westwood, Mass) with exposure to a laser light of 488 mm at the intensity of 50 mW as described previously.[11] The results were expressed as the relative proportions of IF-positive cells Detection of human T cell leukemia/lymphotropic virus type I (HTLV-I) proviral DNA has been performed using a provirus clone, ATK-1, as a source of the probe.[15,16]

Isolation of HTLV-I particles and envelope protein of HTLV-I. HTLV-I particles were obtained from 2 L of culture medium of MT-2 cells as described by Yoshida et al[15] with a slight modification. Briefly, HTLV-I particles were obtained by centrifugation at 25,000 rpm for 90 minutes in a Hitachi RP 42 rotor after removing cell debris by low centrifugation (2,000 rpm for ten minutes). Crude HTLV-I particles were suspended in TNE buffer (20 mmol/L Tris HCl, pH 7.5/100 mmol/L NaCl/1 mmol/L EDTA), layered on a sucrose density gradient (20% to 60%), and centrifuged for 18 hours at 25,000 rpm in the rotor. For analysis of viral proteins, aliquots of each fraction were subjected to 15% polyacrylamide slab gel electrophoresis as described by Laemmli.[17] A low-molecular-weight kit from Pharmacia was used for molecular weight markers. Proteins were stained by silver staining methods. Fractions that contained virus proteins were collected, dialyzed against PBS, and stored at −70 °C until use. The concentration of virus is arbitrarily set as described below; 1 unit of virus per milliliter corresponds to 1.0 at 260 nm (A_{260}).

Envelope protein of HTLV-I was obtained by transforming *Escherichia coli* strain JK1046 with plasmid PEH9 The protein was purified as described previously.[18]

Cell surface marker analyses. Cell surface markers were analyzed by a laser flow cytometry system as described Malignant cells were cultured in 24-well tissue culture plates (Linbro Flow Lab, McLean, Va) at a concentration of 2×10^6 cells per milliliter in a total volume of 1 mL. OKT3 mAb (which was dialyzed against PBS for 18 hours at 4 °C), PMA, and Con A were added at various concentrations. After 18 hours of culture, T3 and Tac antigens were examined by indirect and direct IF methods.[11,12] For direct IF assay, the cutoff point was determined for each sample at which 0.5% of non-stained cells were positive, and the percentage of IF-positive cells was determined. Cells cultured with IL 2 were examined using the same system. The saturating amount of antibodies was determined using T8-positive chronic lymphocytic leukemia cells (T-CLL) for the OKT3 mAb indirect IF system and HUT 102 cells for the TAC mAb direct IF system.

RESULTS

Patients. Clinical profiles of the patients are presented in Table 1. Sera from all ATL patients were positive and those from non-ATL patients were negative for both anti–ATLA-Ab and anti–MA-Ab. Proviral DNA was demonstrated in all ATL cells and not detected in non-ATL cells. The diagnoses of chronic or acute ATL were based on clinical profiles as described elsewhere.[19] Surface phenotype analyses of cells immediately after cell separation showed that all ATL cells were T3$^+$, T4$^+$, T8$^-$, Tac$^+$; cells from patients MG, NB, and IT were T3$^+$, T4$^+$, T8$^-$, Tac$^-$; cells from patient ST were T3$^+$, T4$^-$, T8$^+$, Tac$^-$. The relative proportions of malignant cells in cell suspensions were assessed either by OKT4 or OKT8 mAb. More than 90% of cells from patients with ATL and T4 non-ATL reacted with OKT4 mAb, and 94% of cells from patient ST reacted with OKT8 mAb. The viability of cells immediately after cell separation was more than 95% and that of cultured cells was more than 90% except for cells cultured with PMA (10^{-8} mol/L); 62% to 90% of these were viable. Cell surface marker analyses of fresh cells are listed in Table 2. All ATL cells expressed Tac antigen and a low MFI of T3 antigen; however, apparent relationships between two markers were not observed. The proportions of all malignant cells bearing T3 antigen and the MFI of T3 antigen did not change significantly during culture for 18 hours in complete medium. The MFI of T3 antigen on ATL cells and on the other malignant cells was 29 to 56 and 77 to 95, respectively (Figs 1A, 2A, and 3A).

Modulation of T3 antigen and induction of Tac antigen of non-ATL cells. In the presence of OKT3 mAb (150 ng/mL), MFI of T3 antigen on normal cells decreased by 74% and those of non-ATL cells decreased by 28% to 54%, depending on the cells (Figs 1A and 4). The relative proportions of T3 antigen-bearing cells decreased by 74%, but those of non-ATL cells decreased only by 2% to 22% (Fig 1B). We also studied OKT4 or OKT8 mAb (150 ng/mL) to deter-

Table 1. Clinical Profiles of Patients

Patient	Age	Sex	Diagnosis	Duration (mo)	WBC ($\times 10^9$/L)	Anti–ATLA-Ab	Anti–MA-Ab (%)	Proviral DNA
MG	61	M	T4-ML (leukemic)	5	16.9	—	4	—
NB	60	M	T4-CLL	4	14.0	—	2	—
IT	59	F	T4-CLL	4	12.0	—	4	—
ST	41	F	T8-CLL	21	10.3	—	3	—
NK	35	F	Acute ATL	2	23.3	+	86	+
IH	44	F	Acute ATL	1	42.5	+	85	+
SW	58	M	Acute ATL	65	12.9	+	100	+
NT	34	M	Chronic ATL	7	8.7	+	85	+
AS	60	F	Chronic ATL	72	21.5	+	95	+
KT	54	F	Chronic ATL	36	9.3	+	97	+
TI	67	M	Chronic ATL	10	19.8	+	98	+
EF	61	M	Chronic ATL	22	11.1	+	100	+

ML, malignant lymphoma, CLL, chronic lymphocytic leukemia, ATL, adult T cell leukemia; anti–ATLA-Ab, antibody to ATL virus-associated antigens; anti–MA-Ab, antibody to membrane-associated antigens.

Table 2. Cell Surface Markers of Fresh Leukemic Cells

	T3		Tac		T4	
	%	MFI	%	MFI	%	MFI
Non-ATL						
MG	94.5	77.2	7.1	21.9	93.4	65.5
NB	94.7	83.4	1.0	23.4	90.7	106.5
IT	92.8	80.2	1.7	20.5	92.5	69.2
ST	97.6	95.4	1.4	29.1	94.2*	166.4*
ATL						
NK	84.5	37.2	35.3	29.9	94.5	62.6
IH	82.5	56.1	39.9	30.2	96.4	98.7
SW	86.9	35.7	46.4	35.7	94.7	86.5
NT	94.3	46.7	38.6	23.0	90.4	51.6
AS	69.0	29.1	9.0	29.9	91.6	78.9
KT	94.3	46.7	38.6	23.0	91.9	70.7
TI	86.0	36.6	29.0	20.7	91.6	51.6
EF	83.5	51.1	21.9	35.0	91.2	48.1

*Indicates reactivity to OKT8 mAb.

mine whether these mAbs modulated T3, T4, or T8 antigens, using T4 (from MG) and T8 (from ST) cells. Cells cultured with these mAbs did not change in reactivity with OKT3, OKT4, or OKT8 mAbs (data not shown). The effects of other mitogens on T3 antigen density were examined. Con A slightly decreased the MFI of T3 antigen on NB T4 cells and PMA markedly decreased those on all cells (Fig 1A). This phenomenon was also observed in cells from ST, in which 90% of the cells were viable after cultivation. The non-ATL cells did not express Tac antigen significantly (0% to 7%), even after cultivation with medium, and did express it after stimulation with OKT3 mAb, Con A, or PMA; and T8 leukemic cells expressed Tac antigen only in the presence of PMA (Figs 1B and 4). MFI and Tac antigen-positive cells also increased in the presence of mitogens used (Fig 1A). In general, the decrease in MFI of T3 antigen was well-correlated with the increase of relative proportions of Tac antigen-bearing cells. Cells cultured with HTLV-I did not change their reactivities to T3 and Tac mAbs at all.

Modulation of T3 antigen and expression of Tac antigen on ATL cells. The results of acute ATL and chronic ATL are shown in Figs 2 and 3, respectively. The changes in MFI of T3 antigen on ATL cells by OKT3 mAb were not as great as those of non-ATL cells (Figs 2A, 3A, and 4). In particular, cells from patient SW did not significantly decrease in MFI of T3 antigen when cultured with OKT3 mAb (Figs 2A and 4). The subtle changes in MFI of T3 antigen on ATL cells could not be attributed to contamination with normal T cells because cells from patient IH, whose peripheral blood leukocyte counts were highest and whose percentage of T4-bearing cells was 96%, had the greatest change in MFI of T3 antigen caused by OKT3 mAb (Fig 2A). In contrast to the non-ATL malignant T cells, changes in MFI of T3 antigen on ATL cells by Con A did not occur. The viabilities of ATL cells cultured with PMA (10^{-8} mol/L) were 62% to 82% and the percentage of Tac antigen-bearing cells decreased (Fig 2B), but MFI of Tac antigen-positive cells increased (Figs 2A and 3A). Tac antigen was expressed, to a variable extent, on all ATL cells immediately after cell separation, and the proportions of Tac antigen-positive cells increased after 18 hours of culture (Table 2, Figs 2B and 3B). The enhancement of ATL Tac antigen-bearing cells and increase in MFI of them by OKT3 mAb were also slight as compared with non-ATL but were evident. Cells from SW,

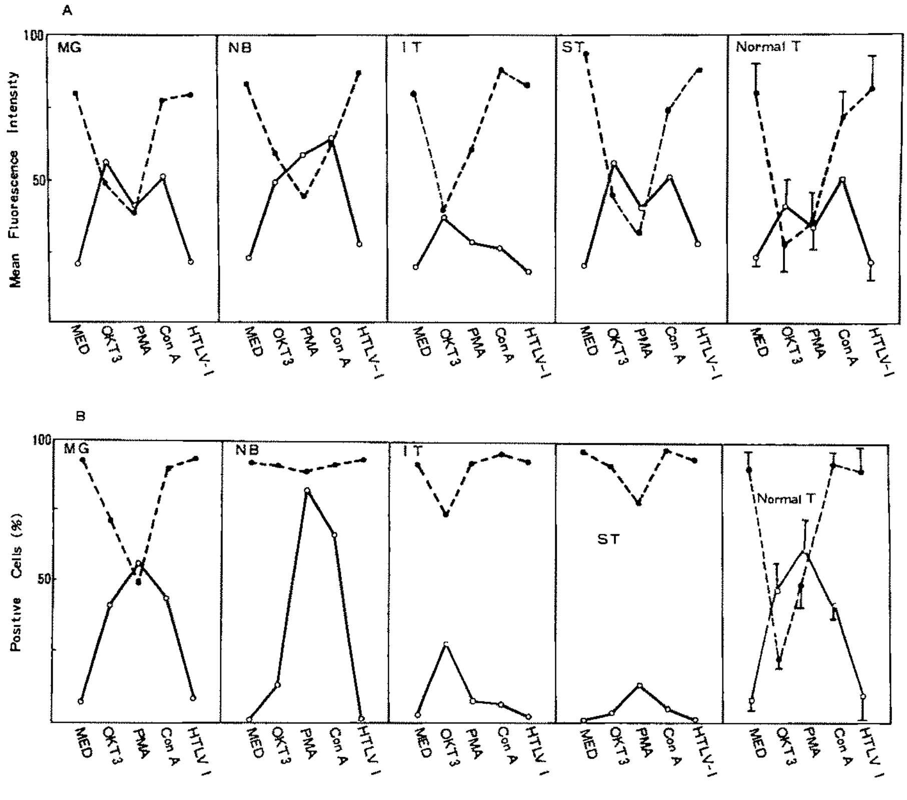

Fig 1. T3 and Tac antigen expression of non-ATL cells after cultivation with various agents. (A) Mean fluorescence intensity of T3 and Tac antigen. (B) Percentage of T3 and Tac antigen-positive cells. Cells were cultured with complete medium, and OKT3 mAb (150 μg/mL), Con A (4 μg/mL), or PMA (10^{-8} mol/L) for 18 hours. After cultivation, cells were stained with OKT3 or anti-Tac mAb and analyzed by flow cytometry. (●), T3 antigen; (O), Tac antigen.

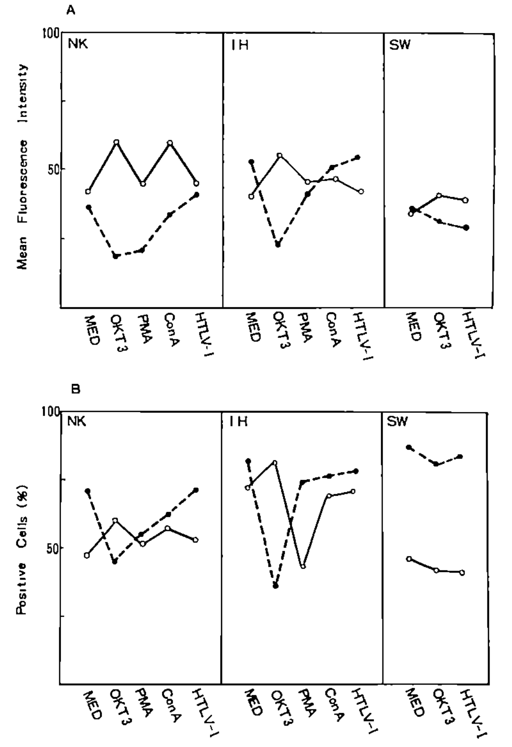

Fig 2. T3 and Tac antigen expression of ATL cells after cultivation with various agents. Mean fluorescence intensity (A) and percentage of antigen-positive cells (B) in acute ATL. (●), T3 antigen; (O), Tac antigen.

whose T3 antigen was not modulated by OKT3 mAb, again did not react with OKT3 mAb with respect to an increase in Tac antigen-bearing cells but, rather, MFI of Tac antigen decreased. T3 antigen on TI cells could be modulated by OKT3 mAb; however, the significant increase of Tac antigen-positive cells was not observed (Fig 3B). When the cells from six other patients whose T3 antigen was moderately modulated by OKT3 mAb (NK, IH, NT, AS, KT, and EF) were examined, the increases in the percentage of Tac antigen-bearing cells and their MFI caused by OKT3 mAb were evident (Figs 2 and 3). Therefore, these two phenomena, the modulation of T3 antigen and the expression of Tac antigen, are closely related, and T3–Ti complex activation can mediate Tac antigen expression on most ATL cells. This suggests that T3 antigen on ATL cells is already modulated in vivo by unknown agents.

T3 and Tac antigens on cultured neoplastic cells. T3 and Tac antigens were examined using cells cultured with IL 2. ATL cells from three patients (NT, TI, AS), non ATL cells from three patients (NB, MG, ST), and normal T cells from three individuals were studied. MFI of T3 antigen increased continuously in all cells examined until day 7, although the percentage of positive cells did not increase (Fig 5). At day 14, decrease in MFI of T3 antigen was observed in one of the ATL cells examined. Interestingly, the percentage of T3$^+$ cells decreased at day 14 in case TI. Percentage of Tac antigen-bearing cells and its MFI on ATL cells continued to increase and reached maximum at day 14 (Fig 6). A subtle increase of Tac antigen-bearing cells was observed

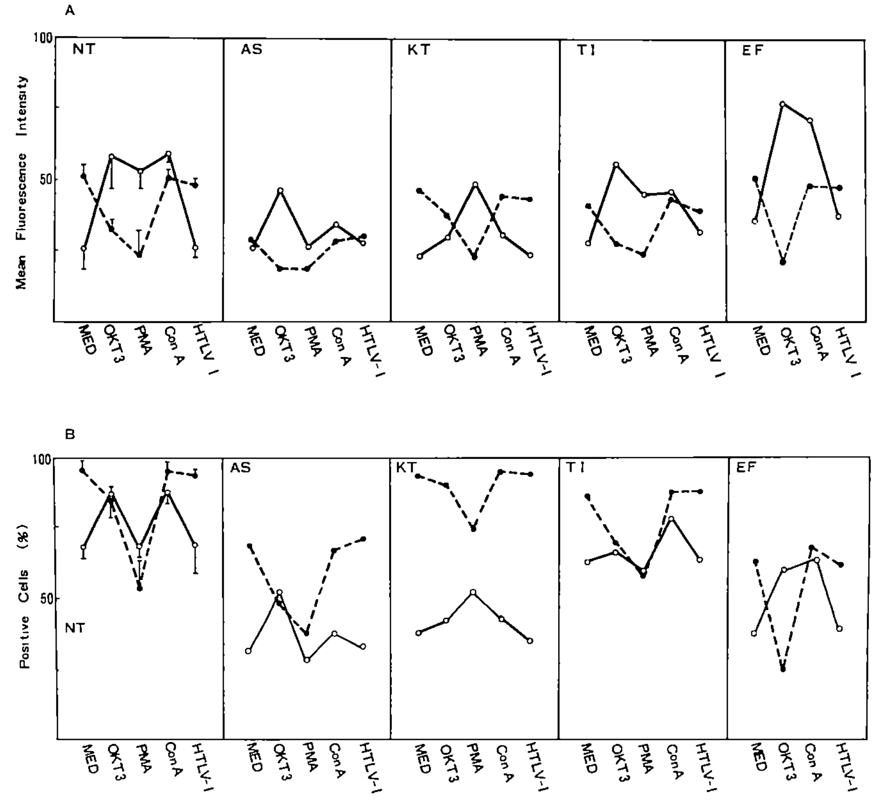

Fig 3. T3 and Tac antigen expression of ATL cells after cultivation with various agents. Mean fluorescence intensity of T3 antigen (A) and percentage of Tac antigen-positive cells (B) of chronic ATL cells.

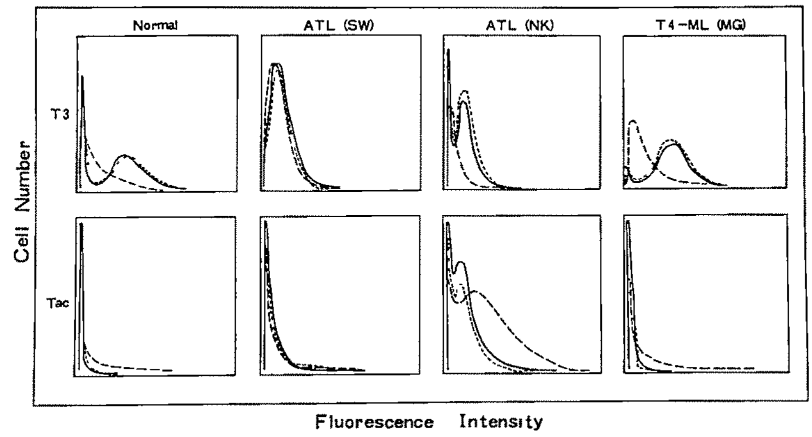

Fig 4. Representitive data of hitogrums of T3 and Tac antigen. Peripheral blood T cells from a healthy person, two ATL patients (SW, NK), and one patient with T4-malignant lymphoma were cultured with medium (—), OKT3 mAb (---), or HTLV particles (· · · ·). After 18-hour cultivation, cells were stained and analyzed as described in Fig 1.

at day 7, when non-ATL cells and normal peripheral blood lymphocytes were used. But the increase was not so marked.

Effect of HTLV-I particles and envelope protein on T3 antigen modulation. It was proposed that leukemogenesis is receptor mediated and that retrovirus interacts with immunospecific or growth receptors of neoplasms.[20,21] So we examined the effects of HTLV-I particles on T3 antigen modulation and Tac antigen expression on all ATL cells. ATL cells were cultured with HTLV-I particles at concentrations of 0.2 to 0.01 U/mL for 18 hours; 0.2 u/mL was chosen as the highest concentration because it saturates HTLV-I receptors on ATL cells as examined by FITC-HTLV particles (unpublished observations, November 1984). At all concentrations, we did not see any significant changes in T3 antigen expression or the expression of Tac antigen on ATL cells from seven cases caused by HTLV-I particles (Figs 2 and 4). We also cultured cells from three patients with ATL (NT, TI, AS) with envelope gene products that have been proven to have antigenicity recognized by serum from ATL patients.[19] The concentrations of envelope protein used were 30, 10, and 3 μg/mL. None of these experiments showed changes of T3 and Tac antigens in any sense. Simultaneously, ATL cells from the same three cases were cultured with 10% patient's serum, which was known to contain antibodies against HTLV-I envelope protein,[18] for seven days to determine whether the serum may prevent the binding of viruses to ATL cells, resulting in the increased expression of T3 antigen. However, the cells cultured with the serum did not change their reactivities to T3 and Tac mAb as compared with medium cultured cells (data not shown).

DISCUSSION

We previously reported that the MFI of T3 antigen on ATL cells is low compared with normal T and T8-CLL cells.[11] Here we have shown that malignant T4 cells from three patients whose sera did not contain anti-ATLA-Ab and whose cells did not have proviral DNA of HTLV-I had the normal density of T3 antigen on their surfaces. T3 antigen on ATL cells was relatively resistant to modulation by OKT3 mAb, although T3 antigen on the non-ATL malignant cells was modulated by OKT3 mAb. In addition, the expression of Tac antigen and the marked increase in Tac antigen-positive cells after culture in complete medium were specific to ATL cells. Non-ATL malignant T4 cells expressed Tac antigen modulation of T3 antigen by mAb or after stimulation by mitogens in 18-hour culture.

The expression of Tac antigen and the low density of T3 antigen on ATL cells suggest that T3–TI on ATL cells may be continuously stimulated by unknown agents in vivo. McGrath et al[20,21] proposed the receptor-mediated leukemogenesis hypothesis because murine leukemia virus (MuLV)-

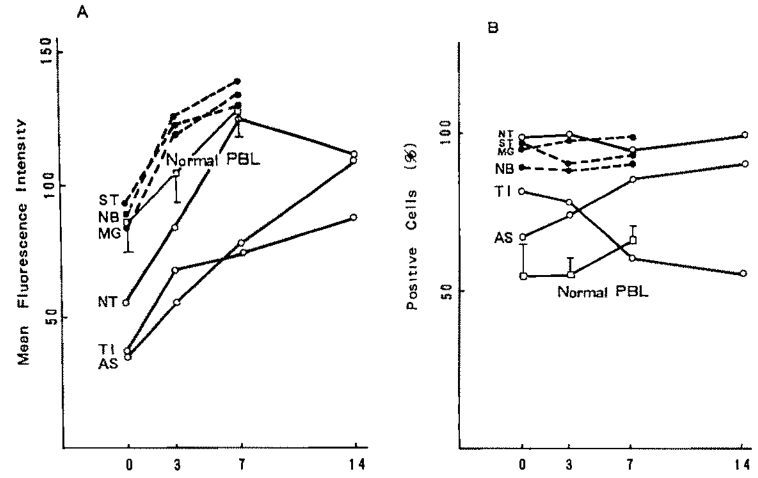

Fig 5. Mean fluorescence intensity of T3 antigen (A) and percentage of T3 antigen-positive cells (B) of cultured cells. (□), mean ± SD of the data of peripheral blood lymphocytes from three healthy individuals; (O), ATL; (●), non-ATL.

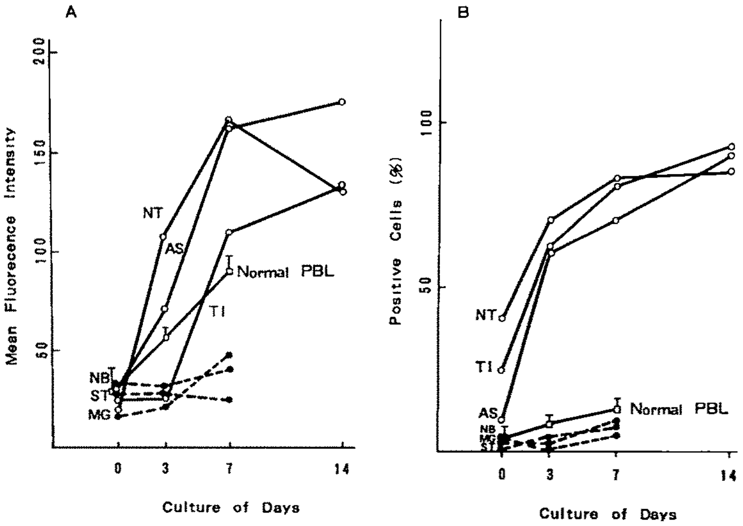

Fig 6. Mean fluorescence intensity of Tac antigen (A) and percentage of Tac antigen-positive cells of cultured cells. Marks used are the same as in Fig 5.

induced thymic lymphoma specifically bound FITC-MuLV, whereas only 0 5% to 2% of normal thymocytes bound them. In this view, the leukemogenic retrovirus express envelope antigens that allow the virus to bind and infect the rare subset of thymic lymphocytes that express receptors complementary to these viral antigens. Thus, it was speculated that slowly transforming retroviruses would interact with immunospecific or growth receptors on the surface of B- and T-cell neoplasms to cause continued rounds of mitogenesis.

Zanders et al[22] reported that the peptides of influenza virus proteins induce the loss of T3 antigen on the surface of cloned T cells specific for influenza virus in 18-hour culture. Therefore, we investigated the effect of HTLV-I particles on the expressions of T3 and Tac antigens on ATL cells to determine whether T3–Ti antigen interacts with HTLV-I protein. The T3 antigen of these cells was not modulated, and there was no enhancement of the expression of Tac antigen on cultivation with HTLV-I particles.

The results indicate that the T3–TI-mediated leukemogenesis hypothesis for ATL cells is less likely. But it is also possible that ATL cells are already tolerant to HTLV-I or other antigens as is suggested by the low density of T3 antigen. Alternatively, envelope protein of HTLV-I may be fragile and cannot react with T3–TI complexes. Thus we used envelope gene products of HTLV-I. Again, no changes in cell surface markers were observed.

It is difficult to interpret the increase of MFI of T3 antigen on cultured ATL cells as well as on non-ATL and normal T cells after cultivation. Increase in MFI of T3 antigen on cultured ATL cells could not be attributed to the selective proliferation of cells that had high density of T3 antigen because it is well known that ATL cells do not proliferate in the presence of IL 2.[23]

There were no differences between acute and chronic ATL cells in our system. However, ATL cells are different from non-ATL T4 malignant cells in MFI of their T3 antigen and in the conditions by which Tac antigen could be induced. Waldmann et al[24] recently reported that ATL cells were Tac positive, whereas most HTLV-negative Sézary cells were Tac negative. Tsudo et al[25] reported that Tac antigen on ATL cells could not be modulated (down-regulation) by anti-Tac mAb, although Tac antigen on Con A-stimulated T cells could be modulated, and proposed that abnormal expression of Tac antigen might play a key role in proliferation of ATL cells. Here we could see increase of MFI or the percentage of Tac antigen-bearing cells by OKT3 mAb as well as PMA, suggesting that Tac antigen on ATL cells were not frozen and could be regulated (up-regulation) by appropriate stimuli. Yodoi et al[26] recently reported a novel lymphokine (ATL-derived factor) that increases the expression of Tac antigen on a human natural killer cell line. A number of other receptors are also related to growth of cells, including transferrin receptor. We have recently observed that transferrin receptors on ATL cells are modulated as those on normal activated T cells (T.U., unpublished observations, August 1984).

Nevertheless, the decrease in MFI of T3 antigen and the expression of Tac antigen were specific to ATL. The expression of Tac antigen on ATL cells was enhanced by T3 mAb stimulation. Thus specific expression of Tac antigen on ATL cells might be the result of the continuous stimulation of T3–TI complexes in vivo by unknown agents.

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Relationship of the Clinical Response and Binding of Recombinant Interferon Alpha in Patients With Lymphoproliferative Diseases

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Patients with hairy cell leukemia (HCL) and chronic lymphocytic leukemia (CLL) were treated with recombinant interferon alpha A (rIFN-αA). The binding of iodinated recombinant interferon-α to baseline samples of peripheral blood mononuclear cells (PBMCs) from the leukemia patients was compared with clinical responsiveness to rIFN-αA. HCL patients (8/10) responded to rIFN-αA therapy, whereas none (0/10) of the CLL patients studied responded. The PBMCs from the eight responsive HCL patients bound approximately twice as much iodinated interferon as the PBMCs from nonresponsive CLL patients. This difference was due to more high-affinity receptors per cell with no difference in the affinity of the interferon-receptor interaction. However, because PBMCs from HCL patients were larger than PBMCs from CLL patients, the cell surface receptor density was similar. The leukemic cells from one of the two nonresponsive HCL patients bound iodinated interferon similarly to the cells from the responsive HCL patients, whereas the leukemic cells from the other nonresponsive HCL patient bound considerably less. The rapidity of response of the HCL patients did not correlate with the level of binding of iodinated interferon. Our results suggest that the absolute number of interferon receptors per cell may be only one of several important parameters in the response to rIFN-αA therapy, and that the responsiveness of a particular lymphoproliferative disease or a particular patient to rIFN-αA therapy cannot be predicted or explained solely by the degree of interaction between IFN and its cell surface receptor.

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PHASE II TRIALS of natural and recombinant interferon alpha preparations have demonstrated clinical efficacy in a number of lymphoproliferative diseases. The most impressive responses have been reported for patients with hairy cell leukemia (HCL),[1] low- and intermediate-grade non-Hodgkin's lymphoma,[2-4] and cutaneous T cell lymphoma.[5] Although early trials with natural interferon alpha suggested activity for chronic lymphocytic leukemia (CLL) patients,[6] a phase II trial with recombinant interferon alpha A (rIFN-αA) demonstrated no appreciable efficacy in CLL patients.[7]

The initial event in the action of interferon (IFN) is the binding to specific cell surface receptors (reviewed in references 8 and 9). The present study was undertaken to determine whether clinical responsiveness to rIFN-αA therapy can be explained, and therefore predicted, by the degree of binding of IFN to its receptor. In this study, patients with HCL and CLL were treated with rIFN-αA. The binding of iodinated recombinant interferon alpha 2 (^{125}I-rIFNα2) and iodinated recombinant interferon alpha A (^{125}I-rIFN-αA) to pretreatment samples of peripheral blood mononuclear cells was measured. Binding data were compared with clinical responsiveness to rIFN-αA.

MATERIALS AND METHODS

Patients The study group comprised patients undergoing rIFN-αA therapy at the Biological Response Modifiers Program's Clinical Unit at the Frederick (Maryland) Memorial Hospital and consisted of ten cases of HCL[10] and ten cases of CLL.[7] All patients in the study were advised of procedures and attendant risks, in accordance with institutional guidelines, and signed informed consent agreements. With the exception of one nonresponsive patient, only those HCL patients with >70% leukemic cells in their peripheral blood were included in the study. All of the CLL patients had 80% to 100% leukemic cells in their peripheral blood. The patients were diagnosed by examination of bone marrow biopsies. Those with HCL had infiltration of the marrow with mononuclear cells containing filamentous cytoplasmic projections and tartrate-resistant acid phosphatase. Those with CLL had at least 30% mature lymphocytes on a differential count of nucleated marrow cells.

Patients with HCL were treated daily with intramuscular (IM) or subcutaneous injections of rIFN-αA at a dose of 3 × 10^6 units.[10] Responses were defined as a normalization of the peripheral blood cell counts and a >50% reduction in leukemic cells in the bone marrow lasting for at least one month.[10] All of the responding patients are still receiving IFN therapy and have had long-lasting responses, with no relapses witnessed to date. Patients with CLL were treated three times weekly with an IM injection of rIFN-αA at doses ranging from 5 to 50 × 10^6 U/m^2 of body surface area.[7] Responses were defined as a >50% reduction in the size of measurable lesions for at least one month.[7]

Cells Leukemic and normal cells were obtained from peripheral blood by venipuncture. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque and washed three times with RPMI 1640 medium with 10% fetal calf serum. Cell viability was >95% for all fresh samples. In some cases, frozen cells were used, cell viability was >80%. No difference was observed between the binding of ^{125}I-rIFN-α2 to fresh or cryopreserved cells.

In some experiments, PBMCs were enriched for hairy cells by depletion of contaminant T lymphocytes. PBMCs (0.3 to 1.2 × 10^8 cells) in 1 mL phosphate-buffered saline with 0.1% sodium azide and 2% human serum albumin (Buffer A) were stained with occasional mixing for 30 minutes at 4 °C with 20 μg fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-Leu-4 antibody (Becton Dickinson, Mountain View, Calif). The cells were washed twice with Buffer A and applied to a 7-mL column of Affi-Gel cell-sorting

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beads with covalently linked antibodies to FITC (Bio-Rad Laboratories, Richmond, Calif). After incubating for 30 minutes at 4 °C, the unbound cells were eluted in Buffer A. The unbound cells were washed with RPMI with 10% fetal calf serum. For analysis by flow cytometry, the cells were washed and incubated for two hours at 4 °C in RPMI without serum. Flow cytometry was performed as previously described[10] with the following monoclonal antibodies from Becton Dickinson: anti–Leu-4 (pan-T lymphocytes), anti–Leu-12 (B lymphocytes), anti–Leu-11 (natural killer cells), and anti–Leu-M_3 (monocytes).

The B lymphoblastoid Daudi cell line was cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated horse serum, as previously described.[11] Small resting T lymphocytes were separated from total PBMCs of normal donors by nylon wool depletion of contaminant adherent cells followed by centrifugation through Percoll gradients, as described[12]; fractions 5 and 6, which contained >90% T lymphocytes, were used for these studies.

Interferon. Recombinant IFN-αA was prepared by recombinant DNA technology and purified with monoclonal antibodies by Hoffmann-La Roche, Nutley, NJ, in collaboration with Genentech, San Francisco. Purified rIFN-α2, obtained from Schering-Plough, Bloomfield, NJ, and rIFN-αA were iodinated, as previously described,[13] with an initial specific activity of 10 μCi/μg and 47 μCi/μg, respectively, based on bindable cpm per microgram of protein active in an antiviral assay. Recombinant IFN-αA and rIFN-α2, which are the products of genes cloned by two groups,[14,15] differ only in a single amino acid, lysine *v* arginine, respectively, at position 23, and are considered to be the same IFN subtype. ^{125}I-rIFN-α2, ^{125}I-rIFN-αA, and rIFN-αA were titered, using an assay for reduction of the cytopathic effect of vesicular stomatitis virus on WISH cells, and activity was expressed as units of IFN by comparison with the international standard Ga23-902-530 for IFN-α. Units of biological activity obtained were converted to nanogram IFN, using the specific activity of pure rIFN-αA (2×10^8 U/mg protein).

Binding assays. Binding assays and competitive displacement experiments were performed similarly to those previously described, using cultured cell lines.[13] For standard binding assays in the present experiments, 3×10^6 to 1.1×10^7 cells in 0.5 mL RPMI 1640 medium with 5% fetal calf serum were incubated with 1 ng ^{125}I-rIFN-α2 or 0.3 ng ^{125}I-rIFN-αA for 1.5 hours at 4 °C. In competitive displacement experiments, increasing amounts of unlabeled rIFN-αA were added to standard binding assays. At the end of the incubation, the samples were layered over 0.2 mL of a 2:1 mixture of di-*n*-butylphthalate:dinonylphthalate in microfuge tubes at 4 °C, as previously described,[13] and centrifuged for two minutes at 10,000 g. The supernatant was aspirated, and the microfuge tube tip, which contained the cell pellet, was counted in a gamma counter (Beckman, Fullerton, Calif) with 40% efficiency. A blank reaction, containing no cells, bound less than 50 cpm over the machine background; this blank was subtracted from all bound cpm reported. The binding of ^{125}I-rIFN-α2 in the presence of 133-fold excess of unlabeled rIFN-αA was defined as nonspecific binding and was subtracted from total binding for Scatchard analysis. Nonspecific binding defined in this way constituted 10% to 20% of the total binding.

Cell sizing. Cell volume distributions were measured with a Celloscope (Particle Data, Elmhurst, Ill), which was standardized with 10.25-μm-diameter polymer latex beads. The data were plotted and analyzed by computer to determine the mean cell volume in each cell preparation.

RESULTS

Eight out of ten of the patients with HCL evaluated in the present study responded to rIFN-αA therapy with dramatic improvement in their hematologic parameters, as shown by normalization of the number of erythrocytes, white cells, and platelets.[10] Surface marker studies with monoclonal antibodies demonstrated a significant decline in the TAC-positive, B cell antigen-positive cells after rIFN-αA therapy. In contrast, none of the CLL patients included in this study responded to rIFN-αA.[7]

The binding of ^{125}I-rIFN-α2 was measured to PBMCs from normal donors and to pretreatment samples of PBMCs from nonresponsive CLL patients and from responsive HCL patients having >70% circulating leukemic cells. The mean cpm bound per 10^7 cells in standard binding assays is shown in Table 1. The mean binding of ^{125}I-rIFN-α2 to PBMCs from CLL patients was only slightly higher than the binding to PBMCs from normal donors. However, the mean binding to PBMCs from HCL patients was more than two times higher than the binding to PBMCs from CLL patients (Table 1). The binding of ^{125}I-rIFN-α2 to the lymphoblastoid B cell line Daudi is shown in Table 1 for comparison. This cell line is highly sensitive to the antiproliferative effect of IFN in vitro[16] and expresses high levels of receptors for IFN-α.[11,13]

Because the binding of IFN at 4 °C is a cell surface event,[17] the data can be expressed as cpm bound per equivalent cell surface area. Therefore, cell volume distributions and mean cell volume in the PBMC preparations were measured. Representative sizing profiles of PBMCs from a normal donor, a CLL patient, and a HCL patient are shown in Fig 1. As shown in Table 1, the mean volume of the PBMCs from HCL patients was almost two times greater than the mean volume of the PBMCs from CLL patients and from normal donors. Making the assumption that the cells were perfect spheres, the mean binding per equivalent surface area was 30% lower for PBMCs from patients with CLL than from patients with HCL. However, because hairy cells have many cytoplasmic projections, and thus are not perfect spheres, the surface area of hairy cells is much greater than calculated here. Therefore, the difference in cpm per surface area between CLL and HCL is even less than estimated by this calculation. The mean binding per equivalent surface

Table 1. Binding of ^{125}I-rIFN-α2 to PBMCs From Normal Donors and From HCL and CLL Patients Before Commencing rIFN-αA Therapy

Cells	No of Patients	cpm/10^7 Cells*	Cell Volume (μm^3)	cpm/10^7 μm^2 Surface Area
PBMCs from responsive HCL patients	8	900 ± 163	330	3.90
PBMCs from nonresponsive CLL patients	10	400 ± 96	167	2.72
PBMCs from normal donors	6	340 ± 102	170	2.28
Daudi cells		4,110	1,216	7.47

The binding of ^{125}I-rIFN-α2 to PBMCs and Daudi cells and the mean cell volume were measured, as described in Materials and Methods. The cell surface area was calculated from the volume, assuming that the cells were perfect spheres.

*Mean cpm bound ± SD

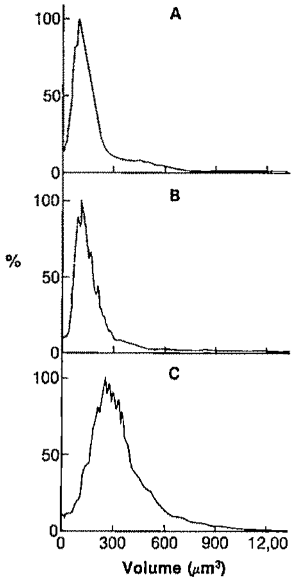

Fig 1. Cell volume analysis of PBMC preparations. Cell volume distributions, determined with a Celloscope, are shown for representative PBMC preparations from a normal donor (A), a patient with CLL (B), and a patient with HCL (C). The number of cells per channel is expressed as a percentage of the maximal signal.

area of PBMCs from normal donors was somewhat lower than the binding to either group of leukemia cells (Table 1).

The higher level of binding of ^{125}I-rIFN-α2 to PBMCs from responsive HCL patients than from nonresponsive CLL patients could be due to either more receptors or a higher affinity of the receptor for the IFN. To assess this, Scatchard plots were constructed from competitive displacement binding curves. In these experiments, a trace amount of ^{125}I-rIFN-α2 was incubated with the cells in the presence of increasing amounts of rIFN-αA. A representative competitive displacement binding curve is shown in Fig 2A. Transformation of these data by the method of Scatchard is shown in Fig 2B. All the Scatchard plots from these experiments, with the exception of that obtained with the Daudi cell line, were curvilinear. As previously discussed,[13,18] many experimental conditions can give rise to artifactual curvilinear Scatchard plots. Therefore, the physiological significance, if any, of these apparent curvilinear Scatchard plots is not presently known.

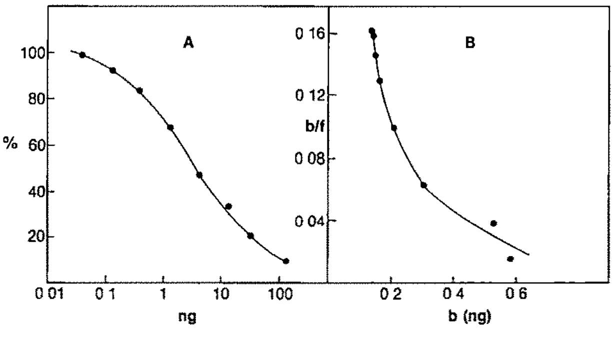

Fig 2. Competitive displacement of ^{125}I-rIFN-α2 by rIFN-αA and Scatchard analysis of the data. PBMCs (1.1 × 10^7 cells) from a patient with HCL were incubated with 1 ng ^{125}I-rIFN-α2 and the indicated amounts of rIFN-αA, as described in Materials and Methods. (A) The binding of labeled IFN is expressed as a percentage of the binding in the absence of unlabeled IFN. (B) The competitive displacement binding data were analyzed by the method of Scatchard with nonspecific binding subtracted, as described in Materials and Methods.

From the high-affinity portion of the curve, the number of receptors per cell and the affinity of the IFN-receptor interaction were estimated. As shown in Table 2, the number of receptors per PBMC from the HCL patients was approximately two times greater than the number of receptors per PBMC from a representative CLL patient. There were fewer receptors on small resting T lymphocytes than on either of the leukemia cell populations. When the number of receptors was expressed per equivalent cell surface area, these differences were substantially reduced. Indeed, as discussed earlier, because of the morphology of the hairy cells, the difference in receptors per surface area is even less than that shown in Table 2. The affinity of the IFN-receptor interaction was the same on all the cells analyzed (Table 2). These results demonstrated that the differences in the binding of ^{125}I-rIFN-α2 to PBMCs from responsive HCL patients compared with those from nonresponsive CLL patients shown in Table 1 could be attributed to differences in the number of receptors per cell with no differences in the affinity of the IFN-receptor interaction.

The data reported thus far compared the binding of ^{125}I-rIFN-α2 to PBMCs from responsive HCL patients with those from nonresponsive CLL patients. However, not all the HCL patients responded to rIFN-αA therapy and the rate of response varied. We compared the responsiveness and the rate of response to therapy with the binding of ^{125}I-rIFN-α2 to PBMCs from HCL patients having >70% circulating leukemic cells. As shown in Table 3, the binding of ^{125}I-rIFN-α2 to PBMCs from these HCL patients ranged from 694 to 1,218 cpm per 10^7 cells, with the exception of patient I, whose cells bound only 207 cpm. After 20 weeks of therapy, HCL patient I did not show any response. The hairy cell count did not decline, which was usually the first sign of response seen within the first week of therapy. In addition, there was no improvement in granulocytes, platelets, or

Table 2. Scatchard Analysis of Competitive Displacement Binding Experiments

Cells	R/Cell*	R/Surface Area†	K_d (mol/L)‡
PBMCs from HCL patients			
A	1,300	510	1.2×10^{-10}
H	1,000	450	1.3×10^{-10}
PBMCs from CLL patient	530	350	1.0×10^{-10}
Resting T lymphocytes	380	300	1.5×10^{-10}
Daudi cells	6,900	1,250	1.3×10^{-10}

The number of receptors per cell and the affinity of the IFN-receptor interaction were estimated from the high-affinity portion of Scatchard plots constructed from competitive displacement binding data. A representative experiment is shown in Fig 2.

*Number of receptors per cell.

†Estimated number of receptors per 100 μm² cell surface area.

‡Dissociation constant.

Table 3. Comparison of Rapidity of Response of HCL Patients to rIFN-αA Therapy and the Binding of ^{125}I-rIFN-α2 to PBMCs

Patient	Weeks Before Response	cpm/10^7 Cells
A	1	807
B	2	694
C	2	852
D	2	1,064
E	3	784
F	4	1,218
G	7	1,063
H	12	746
I	—*	207

In this comparison, a positive response is defined as a >90% decrease in the absolute number of hairy cells in the peripheral blood All of these patients, except patient I, had a normalization of erythrocytes, granulocytes, and platelets within eight to 35 weeks of initiating therapy. The binding of ^{125}I-rIFN-α2 to PBMCs was measured as described in Materials the Methods.

*No reduction in circulating hairy cells or improved blood counts after 20 weeks.

hemoglobin. Interestingly, this patient had massive splenomegaly, which may have contributed to the lack of detectable response. With the other HCL patients there was no correlation between the cpm bound to PBMCs and the length of therapy before a >90% reduction in circulating hairy cells was observed (Table 3).

After this initial study was completed, PBMCs from a second nonresponsive HCL patient were evaluated for IFN-α receptors. However, because only 28% of the circulating PBMCs from this patient were leukemic, it was necessary to enrich for the hairy cells. As described in Materials and Methods, PBMCs were partially depleted of T lymphocytes by cell-sorting affinity chromatography. As shown in Table 4, this procedure reduced the level of Leu-4–positive T lymphocytes and enriched for Leu-12–positive cells; Leu-12, a B cell marker, correlates with the percentage of hairy cells in these patients. This procedure did not appreciably affect the ability of the cells to bind iodinated IFN, as shown for patients A and I. In these experiments, ^{125}I-rIFN-αA rather than ^{125}I-rIFN-α2 was used. Although the absolute number of cpm bound was different, the ratio of binding of ^{125}I-rIFN-αA to PBMCs from patients A and I shown in Table 4 was similar to that previously observed with ^{125}I-rIFN-α2 (Table 3). With both types of iodinated rIFN-α, cells from the nonresponsive patient (I) bound considerably less iodinated rIFN-α than the cells from the responsive patient (A). As shown in Table 4, PBMCs from the other nonresponsive HCL patient (J), when enriched for hairy cells, bound intermediate levels of ^{125}I-rIFN-αA. However, this enriched population of cells was still only 48% Leu-12 positive. Analysis by flow cytometry of unfractionated PBMCs from this patient showed that besides the Leu-4– and Leu-12–positive cells, 12% of the cells were positive for Leu-11, whereas none were positive for Leu-M3, suggesting that there were natural killer cells but no monocytes in the PBMCs of patient J. Therefore, the affinity column-enriched preparation from patient J probably contained large granular lymphocytes (LGLs) in addition to the hairy cells and T lymphocytes. When assayed for the binding of ^{125}I-rIFN-αA under the same conditions as in Table 4, highly enriched preparations of T lymphocytes bound 530 cpm per 10^7 cells and LGLs bound 810 cpm per 10^7 cells. The calculated binding of ^{125}I-rIFN-αA to a cell population consisting of 50% hairy cells (using the cpm bound to affinity column-purified cells from patient A), 25% T lymphocytes, and 25% LGLs was 1,860 cpm per 10^7 cells, which was similar to the binding observed to the hairy cell-enriched preparation from patient J (Table 4). These results suggest that the leukemic cells from nonresponsive patient J bound iodinated IFN at the same high level as the responsive HCL patients, rather than at the low level observed with the other nonresponsive HCL patient I.

Table 4. Binding of ^{125}I-rIFN-αA to PBMCs Enriched for Hairy Cells From Responsive and Nonresponsive HCL Patients

Patient	Response to IFN Therapy	% HC*	Enrichment	Flow Cytometry†		cpm/ 10^7 Cells
				Leu-4	Leu-12	
A	+	88	—	8	80	2,420
			+	3	90	3,060
I	—	96	—	6	96	650
			+	3	94	480
J	—	28	—	54	19	NT
			+	24	48	1,840

Positive and negative responses are as defined in Materials and Methods. PBMCs were analyzed before (—) and after (+) enrichment for hairy cells by T cell depletion as described in Materials and Methods The binding of ^{125}I-rIFN-αA to the cell populations was measured as described in Materials and Methods. NT, not tested.

*Percentage of PBMCs that are hairy cells as determined by morphology

†Percentage of positive cells.

DISCUSSION

In the work described here, we have examined whether the clinical responsiveness or nonresponsiveness of lymphoproliferative diseases to rIFN-αA therapy can be explained, and therefore predicted, by the level of binding of IFN to its cell surface receptor. Patients with HCL and CLL were studied. Eight out of the ten HCL patients included in the study have responded to rIFN-αA therapy as previously described.[10] Similar responses for HCL patients treated with rIFN-α2 have been reported.[19,20] The patients with CLL showed a poor response.[7]

PBMCs from responsive HCL patients bound more than twice as much ^{125}I-rIFN-α2 as the PBMCs from nonresponsive CLL patients. It should be mentioned that in these studies, PBMCs from HCL patients were more than 70% leukemic, whereas PBMCs from CLL patients were nearly 100% leukemic. The major contaminating cells in the peripheral blood from CLL patients were neutrophils, which were removed by Ficoll-Hypaque treatment. The major contaminating cells in the PBMCs from HCL patients were T lymphocytes and LGLs. Because, as discussed in Results, these contaminating cells bound considerably less iodinated IFN-α than did hairy cells, their presence resulted in an underestimation of the binding to hairy cells, making the differences in binding between pure populations of HCL and CLL even greater than shown in Table 1. The more than twofold difference between the binding of iodinated IFN-α to PBMCs from HCL and CLL patients was attributable to more high-affinity receptors per cell with no difference in the affinity of the IFN-receptor interaction. However, we found that the cell volume of the PBMCs from HCL patients was approximately twice that of the PBMCs from CLL patients. Moreover, the hairy leukemia cells had many cytoplasmic projections, which further increased their cell surface area. Therefore, our results indicate that the PBMCs from HCL patients have a greater absolute number of high-affinity IFN-α receptors per cell than do PBMCs from CLL patients, but that they have a similar cell surface high-affinity IFN-α receptor density due to differences in cell size and morphology.

We asked whether the twofold difference between HCL and CLL patients in the absolute number of high-affinity IFN-α receptors per PBMC could account for the responsiveness of the HCL patients and nonresponsiveness of the CLL patients to rIFN-αA therapy. It is possible that if a threshold number of interactions between IFN and its receptor is required for an antiproliferative response, the absolute number of receptors per cell, rather than receptor density, is an important parameter in determining a response. Although in vitro antiproliferative responsiveness may not necessarily correlate with clinical responses, the available data on the Daudi cell line is consistent with the above hypothesis, since these cells express a high number of IFN-α receptors[11,13] and are sensitive to the antiproliferative effects of this IFN.[16] Moreover, in some cases, a reduced responsiveness or lack of response to the antiproliferative effects of IFN has been correlated with a low level of IFN receptors.[21,22] With other cell lines there is no direct relationship between the number of receptors per cell and the responsiveness to IFN.[23,24]

Although the data with HCL and CLL patients suggested that the absolute number of IFN receptors per cell may be an important parameter, the following observations provided evidence that this is not a sufficient criterion to explain or predict a clinical response or the rapidity of response to rIFN-αA therapy. First, among the responding HCL patients, there was no correlation between the level of ^{125}I-rIFNα2 binding and the rapidity of response to rIFN-αA therapy. Second, an enriched preparation of hairy leukemic cells from one of the two nonresponsive HCL patients, when normalized for percentage of leukemic cells, bound iodinated IFN-α similarly to the leukemic cells from the responsive patients. This patient was removed from study after eight weeks due to progressive disease. Although the leukemic cells from the other nonresponsive HCL patient bound the lowest amount of iodinated IFN-α, it is premature to conclude that the lack of response is due solely to the low IFN binding; however, it may be a contributing factor. This patient showed no improvement after rIFN-αA therapy and was removed from study because of increasing pain from splenic infarcts. Interestingly, both of the nonresponding patients had massive splenomegaly. One hypothesis is that the tumor-infiltrated spleens prevented IFN from saturating receptors. Although higher doses of rIFN-αA might have been effective, we felt that they might have led to unacceptable toxicity and instead elected to treat these two patients with 2-deoxycoformycin. Both patients initially responded to deoxycoformycin therapy with improved blood counts and significant reduction in the size of their spleens. Taken together, the data presented here indicate that the responsiveness of a particular patient to rIFNαA therapy cannot be predicted or explained solely by the degree of interaction between IFN and its cell surface receptor.

At the present time, the molecular mechanism for the antiproliferative state induced by IFN is poorly understood. Using cultured cell lines, it has been shown that after the binding of IFN to its receptor, the IFN–receptor complex is internalized by the cells,[13,17,25,26] resulting in down-regulation or reduced number of IFN receptors at the cell surface.[27] Whether internalization of IFN or its receptor is required for the biological responses to IFN has not been resolved.[13,17,28,29] After several hours, new proteins induced by IFN treatment can be detected in the cells.[30] Two of these proteins, 2′5′ oligo(A) synthetase and a protein kinase, are important in the IFN-induced antiviral state.[31] Whether either of these proteins also plays a role in the antiproliferative response to IFN has not been established.[32] Likewise, the function of the other induced proteins is not known.

Because the molecular mechanism for the IFN-induced antiproliferative state in vitro is so poorly understood, it is difficult to explain why some malignancies have responded well to IFN therapy in vivo and why others have not. It may relate to the fact that some malignancies have a viral etiology, and thus the antiviral action of IFN could play a role. In this regard, a T cell variant of HCL was found to contain HTLV-II,[33] a human retrovirus associated with disease. It is also possible that the responsiveness of a particular malignancy or a particular patient to IFN therapy may lie not only with an effect of IFN on the malignant cells, but also with an effect of IFN on the immune system. Further research is required to elucidate molecular mechanisms of the antiproliferative action of IFN in vitro and in vivo.

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On the Mechanism of Hb F Elevations in the Baboon by Erythropoietic Stress and Pharmacologic Manipulation

By D. Lavelle, J. DeSimone, P. Heller, D. Zwiers, and L. Hall

Maximal fetal hemoglobin (Hb F) elevations in the baboon subsequent to phenyl hydrazine-induced hemolytic anemia, bleeding, bleeding plus hydroxyurea (HU), or cytosine arabinoside were two to three times lower than those achieved with bleeding plus 5-azacytidine (azaC). Because, in the baboon, maximal elevations in F cell numbers occurred with bleeding alone, changes in the levels of Hb F in hemolysates and in Hb F per F cell could be considered to be due to the administered drugs. Erythropoietic toxicity of azaC was minimal, making it unlikely that the marked elevations of Hb F were due to shifts in the population of erythroid progenitors and precursors and more likely that they were related to a biochemical effect of the drug on DNA. The data indicate marked DNA hypomethylation. This was also found to be associated, but to a much lesser extent, with the modest Hb F elevations after bleeding, hemolysis, and treatment with HU. This drug had greater erythroid toxicity than azaC, and it appeared that the Hb F elevations occurred mainly on the rebound from the early cytotoxicity. The explanation of the molecular DNA changes with this drug and in erythropoietic stress alone remains unknown.

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FETAL HEMOGLOBIN (Hb F) LEVELS in baboons increase in response to erythropoietic stress due to bleeding[1,2] and are further augmented by the administration of 5-azacytidine (azaC).[3,4] Before its use in baboons, azaC had been found to enhance gene expression in vitro in several eukaryotic cell systems,[5,6] presumably because of its inhibiting effect on the enzymatic methylation of DNA. Later studies showed that hydroxyurea (HU)[7] and cytosine arabinoside (araC),[8] cytotoxic agents active in the S-phase, and vinblastine, which inhibits mitosis,[9] also increase Hb F levels in primates, although not as markedly as azaC. This suggests that a perturbance of the erythroid progenitor pool contributes to the production of erythrocytes containing various amounts of Hb F (F cells). Our studies[3] on the effect of azaC in baboons, kept anemic by bleeding, indicated that the increase in Hb F occurred in two phases: (1) accelerated erythropoiesis due to erythropoietic stress leads to maximal elevations in the number of F cells up to a genetically determined limit[2] and (2) azaC further increases the concentration of Hb F per F cell without further increase in the number of F cells.[3] The former is explainable by present concepts of the relationship of premature maturation of erythroid progenitor cells and F cell production[10]; the latter seemed best explained by a direct biochemical effect of the drug on γ-gene expression, possibly by way of DNA hypomethylation.[3] Because, in baboons, the maximal number of F cells was observed after bleeding alone, before the use of drugs,[3] an opportunity was offered to compare the effects of azaC, HU, and araC on Hb F levels beyond those attained by bleeding alone. In addition, we attempted to determine whether in the baboon the degree of γ-gene–specific and of genomic hypomethylation in erythroid precursor cells could be correlated with the increased Hb F levels.

MATERIALS AND METHODS

Experimental animals and mode of treatment. The effect of azaC on Hb F production was compared with that of HU or araC in 14 baboons kept anemic by bleeding. In six of these 14 animals, hemolytic anemia was induced by phenylhydrazine (PHZ) to compare the effect of a more severe erythropoietic stress than bleeding with the Hb F production due to the drugs. Eleven additional anemic (by bleeding) animals received only azaC and two only HU, for a total of 27 animals. Bone marrow aspirations were performed for examination of the morphological features and for DNA extraction in almost all animals before and after treatment. Of the 27 baboons, 18 belonged to the species *Papio anubis* and nine to that of *P cynocephalus*. The importance of this distinction lies in the fact that most baboons who respond to erythropoietic stress and azaC with the production of high levels of Hb F belong to the species *P anubis*, and most low Hb F responders belong to the species *P cynocephalus* [11] The age of most animals was two to six years; four were older (2057, 2883, 3200, and 3433) and three, younger (5004, 5005, and 5058).

All animals were bled before drug treatment to reduce the packed red cell volume (PCV) to approximately 20% within five days Variable amounts of blood calculated to maintain the PCV at this level were withdrawn almost daily throughout the experimental period. The first dose of the drug was given not earlier than 12 days after the start of phlebotomies. Of the 14 individual animals in which the effects of different drugs and of PHZ hemolysis on Hb F production were compared, all received 5-azaC; eight also received HU and four, araC. Each treatment started only after complete recovery from the previous one and when all measured hematologic parameters were normal. In the majority of animals, the dosage of azaC was 4 mg/kg/d administered intravenously 5 days a week for two to three weeks. By that time, maximal Hb F levels were reached. Ten of the 25 animals received only 0.5 mg/kg/d together with tetrahydrouridine (THU), 10 mg/kg/d. This agent prevents the rapid inactivation of azaC to 5-azauridine by inhibiting cytidine deaminase and has previously[12] been shown to make the reduction of the dose of azaC possible without interfering with the maximal effect on Hb F production. The dosage of HU was 100 mg/kg/d for an average of 1.5 weeks in eight animals; one (3433) received 25 mg and one (4625), 50 mg. AraC was given at a dose of 4 mg/kg/d for one to four weeks. The duration of treatment with these two drugs was determined by signs of worsening erythropoietic toxicity PHZ was administered intraperitoneally at a dose of 20 mg/kg/d for five days without prior bleeding of the animals.

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Peripheral blood. Complete blood counts, white cell differential, and reticulocyte counts were performed daily throughout the period of observation. Hb F levels were determined daily by alkali denaturation[13] and in some of the hemolysates by high-performance liquid chromatography (HPLC), using a large-pore (300 Å) C_4 reverse phase resin.[14] There was no significant difference in the results of the two methods.

Bone marrow. Bone marrow samples were obtained from both untreated and treated animals after two weeks of bleeding and two to three days after the end of drug treatment. These bone marrows were examined morphologically, and DNA was extracted from the original aspirate in the case of marked (80% to 90%) erythroid hyperplasia or from separated cells Separation of erythroid from nonerythroid cells from normal bone marrow was partially accomplished by Ficoll-Hypaque density-gradient centrifugation and unit-gravity sedimentation.[15] Granulocytes were separated from normal peripheral blood by Ficoll-Hypaque centrifugation.

DNA. DNA was purified from bone marrow cells and from granulocytes of peripheral blood by phenol extraction and proteinase K digestion.[16] The deoxymethylcytosine (dmC) content of the DNA was determined by HPLC on a 4.6 × 250-mm C18 reverse phase column (Altex Ultrasphere) at 35 °C according to described techniques[17] with minor modifications. Isocratic development with 0.02 mol/L KH_2PO_4, 8 5% methanol, pH 7, for eight minutes was followed by a gradient in methanol to 30% in ten minutes. The flow rate was 1 mL/min. The results were expressed as percentage of total deoxycytosine.

For Southern blotting,[18] 10 μg of DNA were digested with a threefold to fourfold excess of the appropriate restriction enzyme overnight at 37 °C. The DNA digestion was monitored by the addition of 0.3 μg of lambda DNA to an aliquot of the digestion mixture. Digested DNA samples were fractionated on 0.8% agarose gels, transferred to nitrocellulose membranes,[18] and hybridized to a nick translated 2.1-kb *Bgl*II-*Bam*HI fragment subcloned in PBR 322, containing the 5′ portion of the human $^{G}\gamma$ gene. Hybridizations were conducted as described by Wahl et al.[19] The membranes were washed three times for 15 minutes each with 2 × standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS), at room temperature, followed by a final stringent wash with 0.5 × SSC and 0 1% SDS, at 65 °C Autoradiography of the membranes was performed for two to four days at −80 °C, using intensifying screens.

RESULTS

Hb F levels. Table 1 shows the maximal values of Hb F in the 14 animals that received, sequentially, azaC and HU or araC. Six were also tested for the effect of PHZ-induced hemolytic anemia on Hb F levels. The highest maximal levels were achieved with azaC. As described previously,[3] the level of Hb F started to increase rapidly after the start of treatment with azaC, and the maximum was usually obtained after the second five-day period (Fig 1C). The maximal Hb F level appeared to be influenced mainly by genetic factors, which determine the difference between high (mostly *P anubis*) and low (mostly *P cynocephalus*) Hb F responders. This difference was also noted with the other treatments. Although age, as seen in animal 3433, which was 8 years old, diminishes the magnitude of the Hb F response to erythropoietic stress, azaC overcomes this restricting influence of age and increases the Hb F level to the genetically determined limit.

The Hb F values in all 25 animals that received azaC are listed in Table 2. The average value in the 17 high Hb F responders was 55.9%; in the low responders it was 33.7%. A few values in the 30% to 40% range overlapped. The distinction between the high and low Hb F response was less sharp after bleeding alone (Table 1) because the animals were of different ages.[2]

In the eight animals that received 100 mg/kg/d HU, the Hb F response was two to three times lower than with azaC (Tables 1 and 3) and the duration of treatment was limited by hematologic toxicity. Typical Hb F responses, as shown in Figs 1A and B, appeared to occur in all instances, with the recovery from this toxicity when the reticulocyte count rose and the bone marrow showed erythroid hyperplasia. The same impression was obtained from the effects of araC, but because of the relatively low levels of Hb F and the predictable toxicity, this drug was used in only four animals. The levels of Hb F after PHZ-induced hemolytic anemia were similar to those achieved by HU; in two animals they were higher.

Table 1. Maximal Hb F Levels (%) in Hemolysates of 14 Individual Baboons in Response to Bleeding, PHZ-Induced Hemolysis, AzaC, HU, and AraC

Animal	Species*	Bleeding	Hemolysis (PHZ)	AzaC	HU	AraC
2057	A	4 3	—	52.4 (2)	37.1 (1)	—
3433	C	<1.0	3 0	27.1 (3)	<1.0 (2)	—
3941	C	<1 0	—	26.4 (2)	10 2 (2)	—
3950	A	6.8	15 4	48.8 (3)	—	15.4
4047	A	3.8	—	30.4 (2)	—	7.1 (3)
4446	A	6 2	—	66 4 (3)	24.6 (3)	—
4447	C	5 7	—	31.4 (3)	—	13.0 (5)
4469	C	<1.0	5.2	37.6 (2)	—	—
4485	A	14 9	31 5	60.5 (2)	22.3 (3)	—
4615	A	6 6	—	72.4 (3)	—	9.0 (1)
4625	A	6.1	—	62.0 (2)	12.9 (3)	—
4778	C	2.4	—	40 1 (2)	10.0 (1)	—
5004	A	17.0	30 3	61.2 (3)	—	—
5005	A	23 1	30.8	73.8 (3)	29.8 (1)	—

Numbers in parentheses indicate weeks (Monday through Friday) of treatment.

*A, *P anubis;* C, *P cynocephalus.*

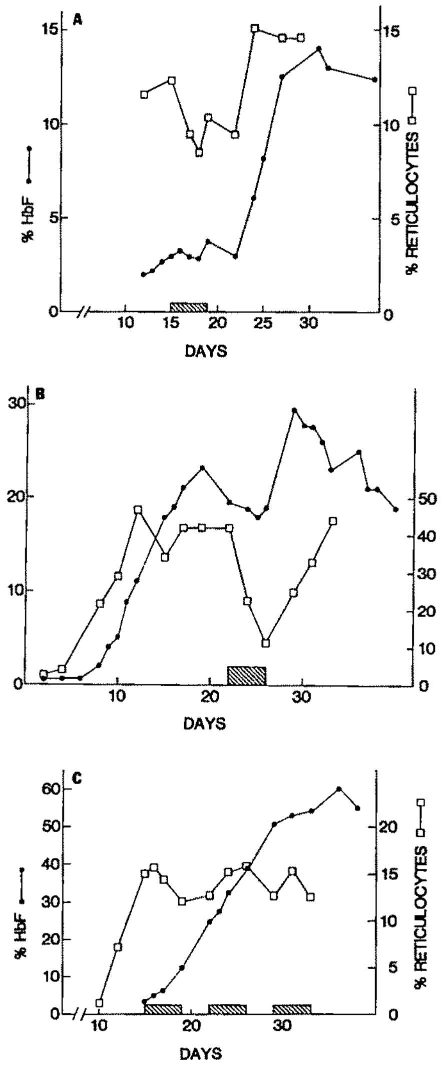

Fig 1. Changes in reticulocyte percentages and Hb F levels of bled baboons after treatment with HU or azaC. (A) Adult animal 8 years old (#2843) treated with HU. (B) One-year-old animal (#5005) treated with HU. (C) Three-year-old animal (#4615) treated with azaC. All animals had PCVs of 20% to 22% maintained by bleeding.

Hematologic toxicity. Tables 2 and 3 show the effect of azaC and HU on reticulocytes and white blood cells. Platelet counts were hardly affected and are not listed. Although all drugs in the administered dosages produced a decrease in the white blood cell count, with seemingly greater influence on granulocytes than on lymphocytes, there were marked differences in their effects on red cell production. AzaC (4 mg/kg/d) changed erythropoiesis very little, as shown by persistent erythroid hyperplasia of the bone marrow and the relatively small fluctuations of the reticulocyte counts, with an average reduction of 8% (Table 2 and Fig 1), whereas HU and araC in the administered dosage decreased the cellularity of the bone marrow and reduced the average reticulocyte percentage by 53% and 36%, respectively. Typical relationships in the time sequence of the reticulocyte count and the Hb F elevations in animals treated with azaC or HU are shown in Fig 1. In the azaC-treated animals, the reticulocyte elevations due to bleeding continued throughout the sequential five-day courses of azaC. In the animals treated with HU, the elevations of the reticulocyte count due to the preceding period of bleeding decreased after the start of treatment and the Hb F level increased only with the return of the reticulocytosis. These consistent differences in the effects of azaC and HU suggest different mechanisms of drug action.

Effect of treatment on γ-gene and genomic methylation. The level of specific methylation in the region of the ${}^{I}\gamma$-gene (analogous to human ${}^{G}\gamma$)[11] was measured by Southern blot analysis of bone marrow DNA samples digested with the methylation-sensitive enzyme *Hpa*II or its isoschizomer, *Msp*I. In a restriction enzyme cleavage map of the baboon ${}^{I}\gamma$-gene (Fig 2), a *Hpa*II site exists immediately 5′ to the coding region of the gene and another such site is located within the large second intervening sequence. In Southern blots of *Bgl*II-*Hpa*II or *Bgl*II-*Msp*I, doubly digested DNA samples hybridized to the 2.1-kb *Bgl*II-*Bam*HI human ${}^{G}\gamma$-probe. Hybridization to a 1.5-kb band was observed, provided that the site immediately 5′ to the coding sequence was hypomethylated. Hybridization occurred to a 0.8-kb band if both *Hpa*II sites were hypomethylated. Because a single *Hpa*II site is involved in the generation of the 1.5-kb fragment, changes in the intensity of this band in Southern blots provide a sensitive means for determining the degree of methylation at this locus. Digestion with *Bgl*II alone resulted in hybridization to a 14.5-kb band.

The extent of γ-gene methylation observed in bone marrow DNA samples obtained from animals undergoing various drug treatments is shown in Fig 3. The 1.5-kb band was found to be visibly more intense in bone marrow DNA samples obtained from animals treated with HU (lane 1), PHZ (lane 3), or bleeding alone (lane 7) than in DNA samples from normal bone marrow (lane 5). The 0.8-kb band was also more intense in bone marrow DNA digests from bled and PHZ-treated animals. Thus these various treatments that resulted in increased Hb F levels were also associated with hypomethylation of *Hpa*II sites surrounding the ${}^{I}\gamma$-gene. The same animal (5004) was the source of DNA samples before (lane 5) and after (lane 3) treatment with PHZ. The most striking increase in intensity of the 1.5- and 0.8-kb bands was observed in bone marrow DNA from animals treated with azaC (lane 9). Thus treatment with azaC, which produced the largest increase in Hb F, also resulted in extensive DNA hypomethylation.

Different patterns of hybridization were found in the blots of *Bgl*II–*Msp*I digests of DNA from bone marrow of HU, PHZ, unbled and bled animals (Fig 3, lanes 2, 4, 6, and 8) compared with DNA from azaC-treated animals (Fig 3, lane 10). The 1.5-kb band was the major band observed after azaC treatment, whereas the others showed only a minor band at 1.5 kb. In these samples, a major band was present at 2.3 kb. Failure to observe a major 1.5-kb band in *Bgl*II–*Msp*I digests except when the DNA has been extensively hypomethylated by azaC was probably due to the inability of

Table 2. Hematologic Responses of All Baboons Treated With AzaC

Animal (N = 25)	Species*	Maximal Hb F	Reticulocytes ($10^5/\mu L$)		WBC			
					Pre		Nadir‡	
			Pre	Post†	$10^6/\mu L$	% Neut	$10^5/\mu L$	% Neut
2057	A	52.4	1.9	1.5	—	—	—	—
3200	A	34.3	3.9	3.0	14.2	26	5.1 (5)	32
3433	C	27.1	1.8	1.4	13.5	91	1.5 (3)	29
3941	C	31.1	4.6	4.5	9.1	68	2.5 (14)	60
3950	A	48.8	2.5	2.0	—	—	—	—
4005	A	67.3	5.9	3.9	—	—	—	—
4047	A	30.4	2.6	2.5	5.3	31	3.5 (5)	13
4048	A	39.0	2.4	2.3	4.4	47	2.8 (5)	11
4430	A	35.8	3.7	4.0	5.7	51	3.9 (8)	49
4445	C	42.2	2.7	3.3	7.6	46	2.7 (5)	33
4446	A	48.0	4.0	4.1	4.6	66	2.0 (7)	38
4447	C	31.4	2.5	2.4	6.6	20	3.8 (1)	6
4448	C	34.4	3.4	4.3	10.0	55	3.3 (12)	31
4469	C	37.6	4.9	3.9	5.4	39	3.5 (6)	23
4485	A	60.5	4.5	2.8	4.6	61	3.4 (9)	39
4612	A	53.0	4.4	3.1	9.7	29	8.7 (10)	37
4615	A	72.4	4.5	4.5	—	—	—	—
4625	A	62.0	3.7	3.0	6.4	43	2.9 (5)	49
4626	A	66.0	3.5	3.3	6.1	52	3.9 (1)	30
4755	C	26.0	4.2	3.7	7.2	31	3.8 (6)	39
4756	A	77.0	3.0	4.2	6.0	48	3.5 (18)	36
4778	C	40.1	2.0	3.4	7.6	54	4.1 (3)	37
5004	A	61.2	4.2	3.7	5.9	63	1.6 (15)	3
5005	A	73.6	3.7	3.0	6.7	37	2.2 (14)	28
5058	A	69.1	3.9	3.2	8.3	40	5.9 (7)	26

All animals had a packed red cell volume of 20% to 24% maintained by bleeding

*A, *P anubis;* C, *P cynocephalus.*

†Last day of treatment.

‡Numbers in parentheses indicate days after completion of treatment.

*Msp*I to digest the methylated CCGG sequence immediately 5′ to the $^{I}\gamma$-gene. Blockage of this analogous sequence to *Msp*I digestion by methylation has been reported in humans,[20] and results from the inability of *Msp*I to cleave a GGCCGG sequence when the 3′C in this nucleotide sequence is methylated.[21,22]

Normal bone marrow samples were found to contain approximately 30% erythroid cells, whereas those obtained after bleeding, HU, and PHZ were 80% to 90% erythroid. Therefore, the increased intensity of the 1.5-kb band observed after bleeding, HU after bleeding, and hemolysis by PHZ could be due merely to an increased proportion of erythroid cells in bone marrow aspirates of erythropoietically stressed animals. To test for this possibility, we compared on the same Southern blot the intensities of the 1.5-kb *Bgl*II–*Hpa*II band from the digest of 30 μg of normal bone marrow

Table 3. Hematologic Responses of All Baboons Treated With HU

Animal (N = 10)	Species*	Maximal Hb F	Reticulocytes ($10^5/\mu L$)		WBC			
					Pre		Nadir‡	
			Pre	Post†	$10^6/\mu L$	% Neut	$10^6/\mu L$	% Neut
2057	A	37.1	5.3	1.6	3.3	51	1.4 (3)	38
2843	A	14.7	3.4	1.7	6.9	52	2.9 (2)	40
3433	C	<1.0	4.0	2.2	—	—	—	—
3941	C	10.2	3.3	2.0	7.1	73	3.1 (3)	48
4444	C	3.1	3.4	1.3	6.4	64	4.8 (5)	55
4446	A	24.6	3.0	0.5	5.7	50	2.9 (19)	35
4485	A	22.3	5.0	2.9	8.9	72	3.2 (5)	55
4625	A	12.9	5.1	1.8	8.4	50	2.9 (1)	52
4778	C	10.0	6.4	4.8	6.6	48	3.8 (1)	68
5005	A	29.8	10.0	4.3	6.1	73	3.7 (1)	28

All animals had a packed red cell volume of 20% to 24% maintained by bleeding.

*A, *P anubis;* C, *P cynocephalus.*

†Last day of treatment.

‡Numbers in parentheses indicate days after completion of treatment

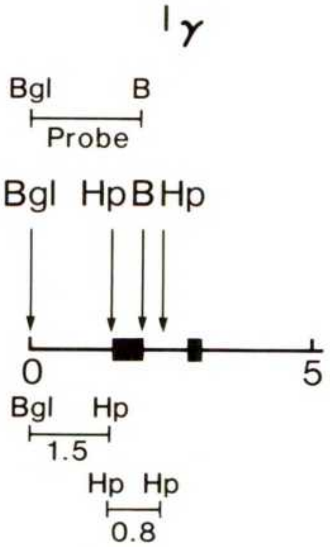

Fig 2. Restriction enzyme cleavage map of the baboon $^{\mathrm{I}}\gamma$-gene. B, *Bam*HI; Bgl, *Bgl*II; Hp, *Hpa*II.

DNA and 10 µg of bone marrow DNA from the same animal after PHZ treatment (Fig 4). The 1.5-kb band of the PHZ sample (lane 4) was at least four to five times as intense as the band observed from normal bone marrow DNA (lane 2), even though only a third of the amount of DNA was loaded into the gel. Thus the observed hypomethylation cannot be explained by a mere enrichment of erythroid cells in the bone marrow samples.

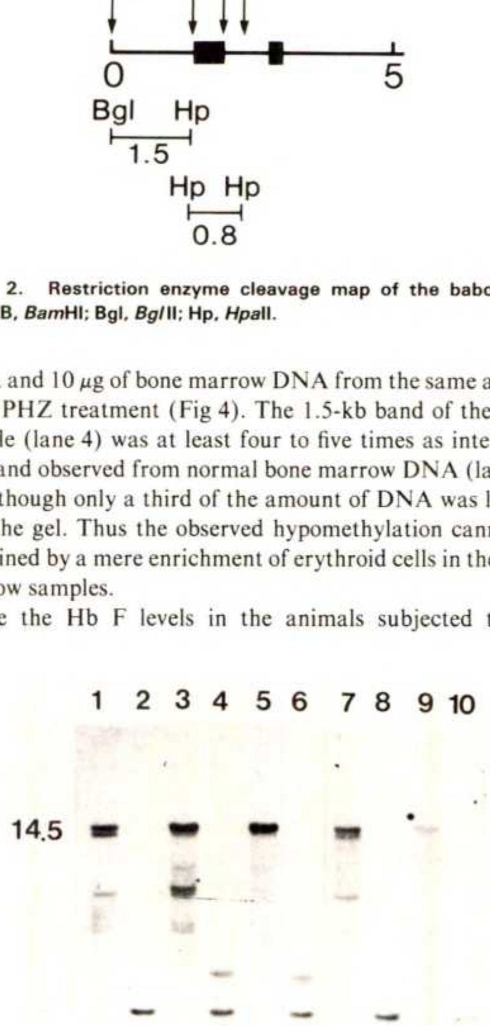

Fig 3. Southern blot analysis of bone marrow DNA restriction enzyme digests hybridized to a human γ-probe (odd numbers: *Bgl*II–*Hpa*II; even numbers: *Bgl*II–*Msp*I): Lanes 1 and 2, Baboon #4778: HU (after bleeding); lanes 3 and 4, #5004: PHZ-induced hemolysis; lanes 5 and 6, same animal: normal; lanes 7 and 8, #5005: bleeding alone; lanes 9 and 10, #4446: azaC (after bleeding).

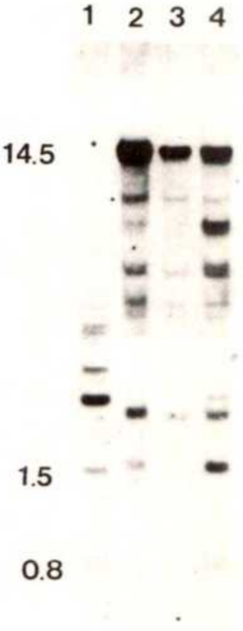

Fig 4. Southern blot analysis of bone marrow DNA (animal #5004) restriction enzyme digests hybridized to a human γ-probe. Lane 1, 10 µg DNA from normal bone marrow digested with *Bgl*II–*MSP*I; lane 2, 30 µg of same DNA digested with *Bgl*II–*Hpa*II; lane 3, 10 µg of same DNA digested with *Bgl*II–*Hpa*II; lane 4, 10 µg of DNA digested with *Bgl*II–*Hpa*II (PHZ-induced hemolysis).

Are the Hb F levels in the animals subjected to the described various treatments proportional to the estimated degrees of DNA hypomethylation? The observations in two animals suggest that this is not the case: Bleeding plus HU treatment of animal 4778 resulted in a maximal Hb F level of 10%, whereas bleeding alone of animal 5005 led to a maximal Hb F level of 23.1% (Table 1). The degree of DNA hypomethylation by Southern blot analysis seemed equal in the two samples (Fig 3, lanes 1 and 7). Thus, in these two animals, the bone marrow DNA was hypomethylated to a similar degree, while the levels of Hb F differed by a factor of 2.3.

We estimated the degree of genomic DNA methylation by determining the percentage of methyldeoxycytosine of total deoxycytosine in the DNA (subsequently designated as dmC content) extracted from bone marrow cells of untreated and treated animals (Table 4). DNA extracted from normal bone marrow, which is approximately 30% erythroid, contained 3.49% ± 0.001% dmC, and DNA from separated bone marrow myeloid cells and peripheral granulocytes contained 3.08% ± 0.007% and 3.01% ± 0.001%, respectively. Thus erythroid cells from normal bone marrow were estimated to contain 4% to 5% dmC. The dmC values of the DNA from erythroid precursor cells of bone marrows with erythroid hyperplasia (80% erythroid), which were obtained from animals after repeated phlebotomies, PHZ, or phlebotomies plus HU were equal at 3% (Table 4) indicating that these treatments were associated with genomic DNA hypomethylation. After azaC, the dmC content was 1.72% ± 0.007%. Thus the lowest levels of genomic DNA methylation were associated with the highest levels of Hb F.

Table 4. Methylcytosine Content of Bone Marrow and Peripheral Blood Cells

	% dmC
Normal bone marrow (N:5)	3.49 ± 0.002
Normal bone marrow myeloid cells (N:3)	3.08 ± 0.007
Normal peripheral blood granulocytes* (N:3)	3.01 ± 0.031
Bone marrow after phlebotomy (N:7)	3.01 ± 0.007
Bone marrow after PHZ (N:4)	3.00 ± 0.001
Bone marrow after HU (N:3)	3.00 ± 0.011
Bone marrow after azaC (N:8)	1.72 ± 0.007

*Granulocytes from peripheral blood and myeloid cells from bone marrow were purified by Ficoll-Hypaque centrifugation and unit-gravity sedimentation.[15] Peripheral blood granulocytes were greater than 90% pure and bone marrow myeloid cells were 75% to 85% pure. Bone marrow of treated animals were at least 80% erythroid. Bone marrow samples were obtained two weeks after the start of daily phlebotomies, one week after start of PHZ injections, two weeks after start of HU and azaC.

DISCUSSION

The data derived from the described experiments in baboons indicate that azaC produces the highest Hb F levels among all experimental manipulations intended to increase these levels, and the highest degree of γ-gene-specific and genomic hypomethylation. The rise in Hb F beginning rapidly after the administration of the drug and the unique magnitude of the Hb F elevations speak for a direct effect of the drug on γ-gene expression in dividing erythropoietic progenitors and precursor cells, including pronormoblasts and normoblasts. Although it is tempting to speculate that DNA hypomethylation precedes the activation of the γ-genes, the exact timing of the two events cannot be determined in such in vivo experiments, as described here.

Recently, Humphries et al[23] also came to the conclusion that azaC has a direct effect on erythropoietic cells on the basis of in vitro and in vivo studies. The authors noted γ-gene hypomethylation in vivo, but because of the Hb F elevations occurring also with HU and araC, they did not attribute any causative significance to this phenomenon. Recent observations of Busslinger et al[24] seem to support the role of hypomethylation in γ-gene expression. These investigators found in in vitro experiments involving the transfer of human γ-gene fragments that methylation of the 5′ end of the human γ-globin gene inhibits the expression of this gene.

According to present concepts,[10] rapid maturation of erythroid progenitor cells, as it occurs in accelerated erythropoiesis, is associated with activation of the Hb F program in these erythroid progenitors, leading to the generation of F cells. Such a mechanism may certainly be set in motion by cytotoxic agents,[25,26] but the remarkably small fluctuations of the elevated reticulocyte count with an average decrease of 8% after treatment with azaC indicate only minor erythropoietic toxicity of this drug at the dosage that leads to the singularly high levels of Hb F. On the other hand, the Hb F elevations that have been found after the administration of HU in patients with sickle cell anemia[27] and in *Macaca fascicularis*[7] and that are not as high as after azaC can be explained by this mechanism. In our experiments with baboons, the Hb F elevations after HU have generally not been higher than in erythropoietic stress, especially if the latter was produced by PHZ hemolysis. These observations suggest that the Hb F elevations after HU depend mainly on the recovery of erythropoiesis after subsidence of the drug's cytotoxic effect. This mechanism seems to apply also to araC[8,28] and vinblastine,[9] which inhibits spindle fiber formation. Although HU is the prototype of a cytotoxic drug, its effect is more complex than is generally assumed, eg, gene amplification by HU has been demonstrated in other cells.[29] It is conceivable that the early appearance of F cells that has been observed 24 to 72 hours after the administration of the drug to patients with sickle cell anemia[27] is due to a transient stimulating effect of the drug on erythropoiesis, but it appears that the change in cell populations during recovery from cytotoxicity is mainly responsible for this phenomenon.

The molecular connection between erythropoietic stress due to bleeding or hemolysis and DNA hypomethylation remains a puzzle. It is conceivable that cellular hyperproliferation produces a similar phenomenon, as has been postulated for the hypomethylation of *Escherichia coli* DNA during amplification in the presence of chloramphenicol,[30] namely, an imbalance between the rate of replication of hyperproliferating cells and the rate of methylation, which are normally closely coordinated. This coordination may be disturbed because the rapid replication leads to a relative deficiency of the rate-limiting DNA methylase. Alternatively, it might be postulated that erythropoietic stress may lead to the amplification of a normally undermethylated erythroid cell subpopulation.

The results of the present study confirm not only the previously described distinction[1] between high and low Hb F responders among baboons, but also the association of these distinctive characteristics with the species *P anubis* and *P cynocephalus*, respectively. This difference is best demonstrable by the Hb F response to azaC. The molecular basis of this difference is not known, but it is independent of the DNA hypomethylating effect of the drug, which was equal in both species.

The favorable influence of Hb F on sickling, provided Hb F and S are in the same cell, and the possibility of increasing Hb F production in β-thalassemia have stimulated the experimental use of azaC and HU in patients with these diseases with favorable results, but their clinical use remains limited because of toxicity and potential carcinogenicity.[25,26,31,32]

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Radiobiological Properties of the Human Hematopoietic Microenvironment: Contrasting Sensitivities of Proliferative Capacity and Hematopoietic Function to In Vitro Irradiation

By Joseph Laver, Wolfram Ebell, and Hugo Castro-Malaspina

We describe the effects of in vitro irradiation on the proliferative capacity and hematopoietic supportive function of human marrow stromal cells. To assess the effects on the proliferative capacity of stromal progenitors and differentiated fibroblasts, marrow cell suspensions and trypsin-dispersed marrow fibroblasts were treated with a single dose of gamma radiation at 100 rad/min. Fibroblastic progenitors (CFU-F) showed an exponential decrease in colony formation with increasing doses of irradiation, with a Do slightly higher than that of granulomonopoietic progenitors (CFU-GM); Do values for CFU-F and CFU-GM were 130 and 115, respectively. However, although the CFU-F survival curve exhibited a shoulder (n = 1.3), the CFU-GM curve did not (n = 1.0), indicating that only fibroblastic progenitors have the potential to repair irradiation-induced damage. Passaged marrow fibroblast colony-forming cells also showed a shouldered exponential survival curve with a Do of 110 and n value of 1.4. Marrow stromal progenitors giving rise to adherent layers in long-term marrow cultures also demonstrated a highly radiosensitive proliferative capacity. Stromal layers derived from irradiated marrow suspensions failed to establish adherent layers after relatively low doses of irradiation (over 240 rad) in a dose–response manner. To assess any functional damage in stromal progenitors surviving irradiation, stromal layers derived from marrow suspensions irradiated up to 240 rad were cocultured with freshly isolated autologous hematopoietic cells and assayed for their capacity to support prolonged CFU-GM production. Confluent stromal layers derived from irradiated marrow suspensions sustained CFU-GM production as well as controls. To study the effects of irradiation on the hematopoietic supportive capacity of established marrow-derived stromal layers, 4 to 6-week-old adherent layers were irradiated as described and cocultured with autologous marrow cells enriched for colony-forming cells. Stromal layers irradiated up to 1,320 rad sustained prolonged CFU-GM production, indicating that the hematopoietic supportive function remained intact at this dose of irradiation. In conclusion, we demonstrated that the proliferative capacity of human marrow stromal progenitors, as well as that of their differentiated descendants, is quite sensitive to in vitro radiation, while the hematopoietic supportive function of differentiated stromal cells is relatively resistant to the effects of radiation.

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THE EFFECTS OF RADIATION on human hematopoietic cells have been extensively studied in the past. For instance, in vitro irradiation experiments have shown that hematopoietic progenitors have a quite radiosensitive proliferative capacity, with Do values (the dose required to reduce survival to 37%) ranging from 90 to 130 rad[1–5] and little or no capacity to repair radiation-induced sublethal damage (no shoulder on survival curves). Similar studies have demonstrated that, with the exception of lymphoid cells,[6–10] most mature nondividing hemic cells are relatively radioresistant in regard to cell viability and some cell functions.[11–13] Analysis of hematopoietic repopulation in patients treated with local irradiation has confirmed in vivo the high radiosensitivity of hematopoietic progenitors and suggested that the failure of hematopoietic reconstitution occurring after local irradiation with doses over 4,000 rad may be related to irreversible damage of the hematopoietic microenvironment.[14–18]

There is overwhelming evidence documenting the essential role of the marrow microenvironment in the process of hematopoietic engraftment and blood cell formation.[19] The fact that intravenously infused hematopoietic cells repopulate the marrow and not other organs of lethally irradiated recipients indicates that this tissue possesses a special microenvironment, which is responsible for the "homing" of primitive hematopoietic stem cells. Evidence for this unique property of marrow stromal cells has been provided by the studies of Friedenstein et al,[20] which demonstrated induction of ectopic hematopoiesis in extramedullary sites by marrow-derived fibroblasts. Furthermore, the necessity of marrow stromal cells for the proliferation and differentiation of early hematopoietic stem cells has been shown in long-term marrow cultures. Prolonged hematopoiesis could not be maintained in the absence of a marrow-derived heterogenous adherent layer, which, as the marrow microenvironment cell population of the intact marrow, is composed of fibroblasts, endothelial cells, adipocytes, and macrophages.[21]

Limited information exists regarding the radiobiological properties of the human microenvironment. In experimental animals, irradiation has been shown to impair the hematopoietic supportive function of marrow stromal cells. Tavassoli[22] showed that irradiation with 500 rad of adherent layers of murine long-term marrow cultures resulted in loss of their ability to support prolonged hematopoiesis. Chamberlin et al[23] noted similar effects using a model of ectopic hematopoiesis. In this study, we have investigated the effects of in vitro irradiation on the survival and hematopoietic supportive function of human marrow stromal cells using the assays for

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fibroblast colony-forming cells (CFU-F) and long-term marrow culture (LTMC), and a coculture assay that represents an in vitro model of marrow transplantation and includes nonirradiated hematopoietic cells and irradiated stromal layers. We demonstrate that the proliferative capacity of human marrow stromal progenitors, as well as that of their differentiated descendants, is quite sensitive to in vitro radiation, while the cell viability and hematopoietic supportive function of differentiated stromal cells are relatively resistant to the effects of radiation.

MATERIALS AND METHODS

Bone Marrow Cells

Bone marrow cells were obtained by aspiration from the iliac crest of healthy volunteers who gave informed written consent. Preservative-free heparin was used as an anticoagulant (Weddel Pharmaceuticals Ltd, London). Marrow buffy coat cells were collected after centrifugation of the aspirates at 200 g for ten minutes. Cells were then washed and resuspended in alpha modification of Eagle's medium (Flow Laboratories, Hamden, Conn) supplemented with 10% fetal calf serum (FCS; Sterile Systems, Logan, Utah).

To obtain marrow cells enriched for hematopoietic colony-forming cells, marrow buffy coat cells were separated into a buoyant and a dense fraction by neutral-density centrifugation in an isotonic sterile Percoll solution (1.074 g/mL, 270 mosm) (Pharmacia Fine Chemicals, Piscataway, NJ) as described elsewhere.[24] The buoyant mononuclear cells were subsequently subjected to two cycles of adherence in T-75 tissue culture flasks, at 37 °C, for one hour. Hematopoietic colony-forming cells contained in the nonadherent low-density (NAL) marrow cell fraction were collected, washed, and resuspended in culture medium

Culture Methods

Fibroblast colony-forming cell assay (CFU-F) The general procedure has been previously described.[25,26] Fibroblast colonies (50 or more cells per aggregate) were scored after ten days of incubation. The fibroblastic nature of cells composing the colonies was demonstrated by immunofluorescence staining with antibodies against fibronectin and types I and III collagen.[25]

Marrow fibroblast cell lines and passaged fibroblast colony-forming cell assay (CFU-F$_p$). Marrow cell suspensions were cultured as for CFU-F and maintained until a confluent fibroblastic layer was formed. Cultures were maintained with medium changes every four days. To passage primary or secondary fibroblast cultures, cell monolayers were washed twice with Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS), covered with an EDTA-trypsin solution (GIBCO, Grand Island, NY), and incubated at 37 °C for ten minutes. Cells were then pipetted off, resuspended in medium containing FCS, washed twice, and subcultured in alpha medium supplemented with 10% FCS.

Colony formation by passaged marrow fibroblasts (CFU-F$_p$) was assessed by the method of Cox and Masson,[27] culturing low numbers of fibroblasts on lethally irradiated marrow fibroblast feeder layers (3,500 rad) Five to 10 × 10^3 cells resuspended in alpha medium supplemented with 20% FCS were inoculated into T-75 tissue culture flasks containing irradiated feeder layers, gassed with 5% CO_2 in air, and incubated at 37 °C. The growth medium was weekly changed Cultures were ended after 14 to 21 days of incubation, and colonies (50 or more cells) were counted after staining with Wright's stain

Granulocyte–macrophage colony-forming cell assay (CFU-GM) Granulocyte–macrophage progenitor cells were assayed using a slightly modified soft agar technique described by Pike and Robinson.[28] Colony formation was stimulated by 10% exogenously supplied colony stimulatory factors present in medium conditioned by human monocytic cell line GCT (GIBCO). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air and scored for colonies (50 or more cells per aggregate) and clusters (three to 50 cells per aggregate) after seven days of incubation.

Long-term marrow culture technique Long-term cultures of human marrow were established according to the method of Gartner and Kaplan[29] with some modifications.[30,31] Briefly, 20 × 10^6 marrow buffy coat cells in 10 mL of LTMC medium were inoculated into T-25 tissue culture flasks. Cultures were then gassed with 5% CO_2 in air and incubated at 33 °C. The LTMC medium consisted of supplemented McCoy's 5A medium containing 12.5% horse serum, 12.5% FCS, and 10^{-6} mol/L hydrocortisone sodium succinate (Calbiochem-Behring, La Jolla, Calif). On day 4 of incubation, in order to eliminate erythrocytes and mature myeloid cells, the nonadherent cells were gently aspirated out of the flasks and enriched for mononuclear cells by Percoll neutral-density centrifugation. The resulting buoyant mononuclear cell-enriched fraction was collected, washed twice, resuspended in 5 mL of LTMC medium, and reinoculated into the original flasks containing the adherent stromal layers and fresh LTMC medium. The latter was added (5 mL per flask) soon after the nonadherent cells were aspirated out of the flasks. At weekly intervals, half the supernatant medium and suspension cells were removed and replaced with fresh LTMC medium. Cells in suspension were counted, checked for viability by trypan blue dye exclusion test, and assayed for CFU-GM.

To assess the kinetics of development of marrow stromal layers, individual LTMCs were examined every seven days under a phase contrast inverted microscope, and the percentage of the flask surface covered by the stromal interlocking network was visually estimated and recorded.

Cocultivation of marrow-derived stromal layers with freshly isolated autologous hematopoietic cells. To assess the capacity of established marrow stromal layers to sustain hematopoiesis in vitro, 4- to 6-week-old primary LTMCs were depleted of all suspension cells, and a second inoculum of freshly isolated autologous hematopoietic cells was added. This consisted of 6 × 10^6 NAL marrow cells resuspended in fresh medium. Cultures were then incubated at 33 °C and subsequently weekly depopulated and fed with an equivalent amount of fresh LTMC medium. Total cell counts and CFU-GM numbers were assayed in the removed cell population. To monitor the endogeneous CFU-GM production in primary cultures, the CFU-GM production in control cultures without added cells was also measured.

Immunocytochemical Analysis of Cultured Marrow Stromal Cells

To evaluate the composition of the stromal layers, we used immunofluorescence (IMF) methods with antibodies directed against components known to be associated with fibroblasts, endothelial cells, and macrophages as previously described.[24,29,30] These studies were performed in situ after the removal of the upper portion of culture flasks with a heated scalpel. Fibroblasts were identified by rabbit antibodies to type III collagen (kindly provided by Dr S Gay, University of Alabama, Tuscaloosa) and human fibronectin (Bethesda Research Laboratories, Gaithersburg, MD), endothelial cells by a rabbit antiserum to human factor VIII-related antigen (Calbiochem-Behring, San Diego), and macrophages by mouse monoclonal antibodies recognizing human monocyte antigens (Bethesda Research Laboratories) Lipid-containing cells were studied with oil red O staining.[32]

Irradiation Procedure

The Gammacell 40 irradiation chamber containing two opposing sources of cesium-137 (Atomic Energy of Canada Ltd, Ottawa) was used to irradiate marrow cell suspensions and cultured marrow stromal layers. The radiation was given as a single dose at the rate of 100 rad/min. During the time of irradiation, control samples were kept outside the Gammacell at the same temperature as the irradiated cultures. Marrow cells resuspended in serum-supplemented growth medium were irradiated with doses up to 600 rad and then assayed for CFU-F, CFU-GM, and LTMC. To assess the effect of irradiation on colony-forming cells, the mean number of colonies formed was used to calculate the plating efficiency and then the surviving fraction relative to the control values. Radiation dose–survival curves were then constructed on semilogarithmic paper Linear regression analyses of the curves were performed so as to yield the parameters of Do and n (the extrapolation number) To assess the effects of irradiation on stromal progenitors giving rise to adherent layers, marrow cells were irradiated and then inoculated for LTMC The kinetics of development and composition of stromal layers as well as cell and CFU-GM production were then weekly determined To assess any functional damage in stromal progenitors surviving irradiation, adherent layers derived from irradiated marrow cells were cocultured with freshly isolated autologous hematopoietic cells and assayed for their capacity to support prolonged CFU-GM production. To study the effects of irradiation on the stromal hematopoietic supportive capacity, established primary heterogeneous adherent layers (4 to 6 weeks old) were irradiated with doses up to 1,320 rad and then cocultured with freshly isolated hematopoietic cells. Subsequently, cell count, morphology, and CFU-GM production of the buoyant fraction were monitored weekly. In another set of experiments, secondary marrow fibroblast cultures were trypsinized, irradiated in suspension, and replated for colony formation in T-75 tissue culture flasks as described. The survival parameters (Do, n) were determined as described.

To assess the potential of cultured marrow fibroblasts to repair radiation-induced damage, we measured the ability of the surviving cells to grow in culture. Passaged marrow fibroblasts were irradiated in suspension and replated. Cultures were then passaged at weeks 1, 2, 5, and 6 after irradiation and plated at the same cell concentration (2×10^5 cells per flask) Between second and third passage, cells were left for a longer period (three weeks) to allow confluency The change in cell number, expressed as percentage of previous passage, was determined.

RESULTS

Effects of Irradiation on the Proliferative Capacity of Marrow Fibroblast and Granulocyte–Macrophage Progenitors

Cell survival curves for CFU-F and CFU-GM after in vitro single-dose irradiation are shown in Fig 1. Each point represents the mean of nine experiments for CFU-F and CFU-GM. Both survival curves show an exponential decrease in colony-forming ability with increasing radiation dose. A small initial shoulder is present only in the CFU-F survival curve. The parameters for survival curves are, for CFU-F, Do:130, n:1.3 and, for CFU-GM, Do:115, n:1.0. The morphology of fibroblast and granulocyte–macrophage colonies growing in cultures of irradiated cells did not differ essentially from that of colonies developing in cultures of control nonexposed cells.

Effects of Irradiation on the Proliferative Capacity of Cultured Marrow Fibroblasts

The cell survival curve for passaged marrow fibroblasts ($CFU\text{-}F_p$) after in vitro single-dose irradiation in cell suspension is shown in Fig 2. Each point represents the mean of three experiments. The survival curve is exponential and shouldered with a Do of 110 and n of 1.4.

The growth pattern of $CFU\text{-}F_p$ after different doses of in vitro irradiation is shown in Fig 2. Results are expressed as the percentage of change per previous passage and represent the mean of three experiments. Control nonirradiated marrow fibroblasts show an increase in number on each passage. Marrow fibroblasts irradiated with 480 and 720 rad exhibited a significant initial decrease in number, followed by rapid proliferation of the surviving cells. Irradiation with doses over 960 rad resulted in a decline of cell number in culture without a subsequent proliferation.

Establishment of Long-Term Cultures with Irradiated Marrow Cell Suspensions

The kinetics of development of marrow stromal layers and the CFU-GM production in long-term cultures derived from irradiated marrow cell suspensions are shown in Fig 3. Nonirradiated marrow cell suspensions were capable of establishing confluent stromal layers by the third or fourth week and of producing CFU-GM for five to seven weeks. Marrows irradiated with doses up to 240 rad established confluent stromal layers but were unable to produce any CFU-GM. A dose of 480 rad markedly impaired the establishment of stromal layers, and a dose of 600 or more rad abolished any stromal layer formation. This impairment could be overcome by a higher inoculum of irradiated marrow cells; the higher the radiation dose, the bigger the inoculum needed to establish a stromal layer (80×10^6 cells for 480 rad and 160×10^6 for 600 rad).

The composition of heterogeneous adherent layers derived from irradiated marrow suspensions is shown in Table 1. Control stromal layers were composed of fibroblasts, adipocytes, endothelial cells, and macrophages, with a clear predominance of fibroblasts. Stromal layers derived from marrows irradiated up to 240 rad had a similar composition to controls. Higher doses of radiation resulted in the establishment of only rare fibroblast islands.

Cocultivation of Stromal Layers Derived from Irradiated Marrow with Autologous Hematopoietic Cells

To analyze the functional capacity of stromal progenitors surviving irradiation, 4- to 6-week-old LTMC with stromal layers derived from irradiated marrows were cocultured with freshly isolated autologous NAL marrow cells. The hematopoietic function of these layers was assessed by quantifying every week the total cell and CFU-GM production. The summarized results of four experiments are shown in Fig 4 (SEM less than ± 10%). Control primary cultures without added fresh marrow cells did not produce any CFU-GM. Production of CFU-GM in cultures containing no stromal layers declined to undetectable levels by week 3. Stromal

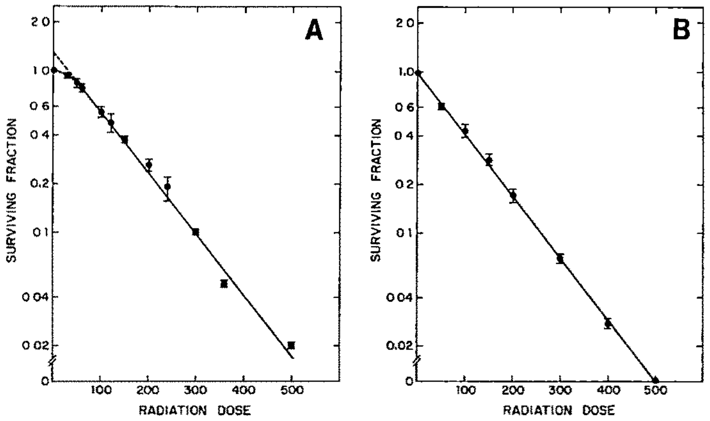

Fig 1. Cell survival curves for CFU-F (A) and CFU-GM (B). Marrow cell suspensions received a single-dose irradiation at the rate of 100 rad/min and were then assayed for colony-forming cells. Results shown represent the mean of nine experiments for CFU-F and CFU-GM. Survival parameters exhibited by CFU-F and CFU-GM were Do:130, n:1.3 and Do:115, n:1.0, respectively.

layers derived from nonirradiated marrow as well as those derived from irradiated marrows with doses up to 240 rad were capable of maintaining CFU-GM production for six to eight weeks. Doses of 480 or more rad severely impaired the establishment of stromal layers, and consequently, no prolonged hematopoiesis in the tissue culture occurred. However, confluent and functional adherent layers could be obtained when a higher inoculum of irradiated marrow cells was used.

Cocultivation of Marrow-Derived Stromal Layers Irradiated After Establishment with Autologous Hematopoietic Cells

To analyze the effects of irradiation on established marrow stromal cells, 4- to 6-week-old established stromal layers derived from nonirradiated marrows were subjected to radiation with doses up to 1,320 rad and then cocultured with freshly isolated autologous NAL marrow cells. The hematopoietic supportive function of these stromal layers was assessed weekly by total cell and CFU-GM production. Figure 5 summarizes the results of five experiments (SEM less than ± 10%). Control irradiated cultures without added fresh marrow cells did not produce any CFU-GM. The CFU-GM production in cultures with stromal layers exposed to 1,320 rad was similar to that seen in the cultures with nonirradiated stromal layers.

The composition of marrow-derived stromal layers irradiated after establishment is shown in Table 1. Irradiation with doses up to 1,320 rad did not affect significantly the composition of these adherent layers.

DISCUSSION

The data presented show that the proliferative capacity of human marrow stromal progenitors, as well as that of their progeny, is quite sensitive to radiation in vitro, while the viability and the hematopoietic function of differentiated marrow stromal cells are relatively resistant to the effects of radiation. The high radiosensitivity of marrow stromal progenitors is demonstrated by the parameters of CFU-F survival and the observations in LTMC showing a significant dose-related inhibition of stromal layer formation. Studies in experimental animals (mice, rabbits, guinea pigs) have shown that marrow fibroblasts are a crucial component of the marrow microenvironment. Cultured marrow fibroblasts transplanted under the kidney capsule generate ectopic hematopoietic microenvironments of donor origin that induce and sustain host-derived hematopoiesis in these sites.[20,33] By affecting the proliferative capacity of marrow fibroblasts, irradiation might damage their renewal potential, and therefore impair the hematopoietic function of the marrow microenvironment. In our study, the close Do values for CFU-F and CFU-GM indicate that fibroblastic progenitors are quite sensitive to in vitro radiation. Also, the

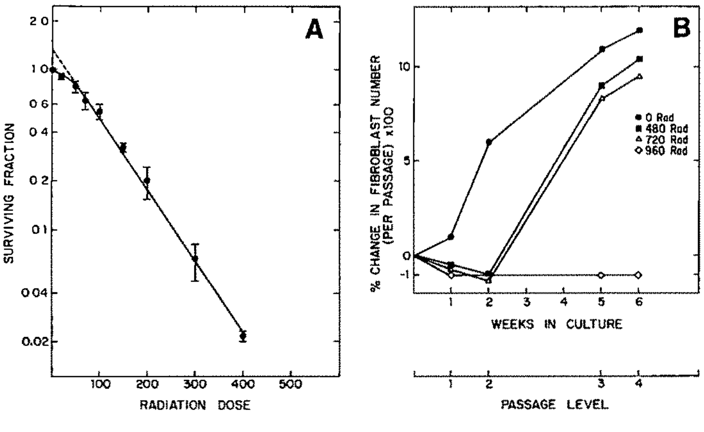

Fig 2. Survival curve (A) of passaged marrow fibroblasts (CFU-F$_P$) and the growth pattern (B) of the surviving cells after irradiation. Passaged marrow fibroblasts were irradiated in suspension and plated for colony formation. Results shown represent the mean of three experiments, showing a Do:110 rad and n:1.4. In another set of experiments, passaged fibroblasts were irradiated and then replated. After weeks 1, 2, 5, and 6, fibroblast number was determined before replating. The increase in fibroblasts number per passage, expressed as percentage of previous passage, was calculated and plotted. The results shown represent the mean of three experiments. Passaged marrow fibroblasts surviving irradiation up to 720 rad were able to proliferate and repopulate the tissue culture flask.

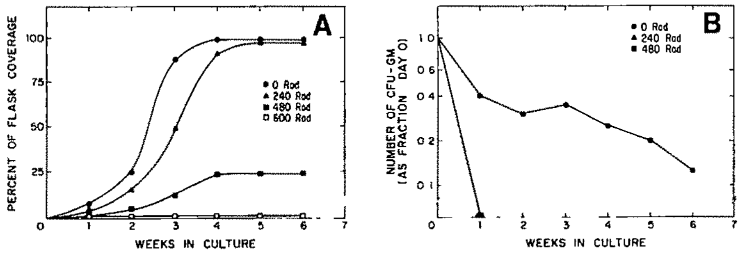

Fig 3. Kinetics of stromal development (A) and CFU-GM production (B) in LTMC derived from irradiated marrow cell suspensions. Marrow cells were irradiated and then inoculated for LTMC at the concentration of 20 × 10^6 per flask. Results shown represent the mean of four experiments. Note that relatively small doses of radiation abolish the long-term production of CFU-GM without impairing significantly the ability to give rise to a heterogeneous adherent layer.

proliferative capacity of passaged marrow fibroblast colony-forming cells (CFU-F_p) is highly radiosensitive. Comparison of our Do values for CFU-F with published data shows that our results are in the reported range.[34,35] Moreover, the Do value for both CFU-F and CFU-F_p is comparable to the Dos of 98 to 160 rad for other human fibroblastic cells of nonmarrow origin.[36] In addition to CFU-F, which represents only one cellular stromal element, we used the LTMC system to assess all the cells constituting the stromal cell population. Stromal cells derived from irradiated marrow cell suspensions failed to establish confluent adherent layers after relatively low doses of irradiation in a dose–response manner paralleling CFU-F and CFU-F_p survival curves. These observations in LTMC clearly show that all stromal progenitors are highly radiosensitive to in vitro irradiation.

The relatively similar in vitro radiosensitivity of marrow stromal and granulomonopoietic progenitors appears to be in discordance with in vivo findings,[14,37] which demonstrate a significantly higher radioresistance of stromal progenitors. Irradiation with doses used in human marrow transplantation ablates hematopoietic cells irreversibly but spares the stromal compartment, as evidenced by the occurrence of autologous stromal regeneration from residual stromal progenitors. An explanation for this apparent discrepancy could be that CFU-Fs are in vivo and in vitro in different phases of the cell cycle with different radiosensitivities. In intact marrow, the microenvironment is not a rapid proliferating compartment[25] in which most of the fibroblastic progenitors are most likely in G_0. In vitro, the growth factors present in the culture medium cause the entry of resting cells (G_0) into the proliferative phase (G_1), which has been shown to be highly radiosensitive.[38] An alternative or concomitant explanation for this discrepancy might be differences in the potential to repair sublethal radiation damage. In favor of this explanation is that CFU-F and CFU-F_p survival curves have a shoulder, which is absent in the survival curves for hematopoietic progenitors. In addition, the more efficient radiation repair mechanisms in vivo[39] may further contribute to the relative radioresistance of stromal cells in intact marrows. These differences in the repair mechanisms must be taken into account when extrapolating from in vitro observations to the cell in the intact organism.

Marrow stromal progenitors surviving in vitro irradiation are able to form normal functioning stromal layers. Coculture experiments have shown that stromal layers derived from irradiated marrow cell suspensions exhibited normal hematopoietic supportive function when a confluent layer was established. Marrow cells irradiated up to 240 rad gave rise to confluent stromal layers with normal hematopoietic support, while higher doses resulted in an impaired establishment. However, using a higher inoculum of irradiated marrow cells, we were able to obtain confluent stromal layers with normal hematopoietic supportive function. Thus a minimum number of surviving cells is required in order to establish a functional stromal layer. The latter finding might explain the process of marrow stromal renewal capacity after irradiation in vivo. The nonproliferative state and the repair potential of stromal cells enable the surviving stromal progenitors to maintain their proliferative capacity and, if an adequate fraction is left, reestablish a normal functioning stroma. Furthermore, experiments with passaged marrow fibroblasts have shown that these cells are able to recover and

Table 1. Cellular Composition of Irradiated Marrow Stromal Cells in Long-Term Marrow Cultures

	Relative Frequency						
	Stromal Layers Derived From Irradiated Marrow Suspensions*				Stromal Layers Irradiated After Establishment†		
Cell Type	Contr	240 rad	480 rad	600 rad	Contr	480 rad	1,320 rad
Fibroblast	4+	4+	1+	±	4+	4+	4+
Adipocyte	2+	2+	0	0	2+	2+	2+
End. cell	1+	1+	0	0	1+	1+	1+
Macrophage	3+	3+	0	0	3+	3+	3+

*Cultures were initiated with 20 × 10^6 irradiated marrow cells per flask. After four to six weeks of incubation, adherent layers were examined for their cellular composition. Irradiation with 480 rad impaired stromal establishment, and after 600 rad, only a few fibroblasts were present in culture.

†Cultures were initiated with 20 × 10^6 nonirradiated marrow cells per flask. Four to 6 weeks postinoculation the adherent layers were irradiated, and 24 hours later their cellular composition was evaluated. The cellular composition was assessed by immunofluorescence staining with antisera against type III collagen and fibronectin for fibroblasts, and against factor VIII-related antigen for endothelial cells, as well as monoclonal antibodies recognizing monocytic antigens, and by light microscopy for adipocytes. The presence and relative amount of different cell types was assessed semiquantitatively by grading their frequency from 0 to 4^+.

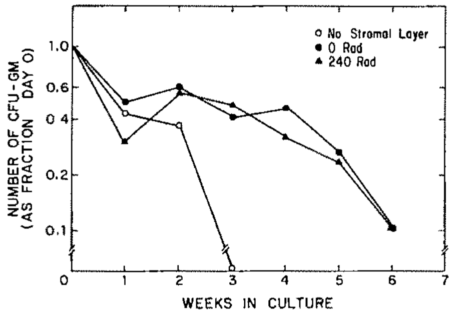

Fig 4. CFU-GM production in cocultures of stromal layers derived from irradiated marrows with autologous hematopoietic cells. Four- to 6-week-old stromal layers derived from 20 × 10^6 irradiated marrow cells were overlaid with 6 × 10^6 freshly isolated autologous NAL marrow cells. CFU-GM production was weekly monitored. Results shown represent the mean of four experiments. Marrow stromal progenitors that survive irradiation gave rise to stromal layers with normal hematopoietic supportive function.

undergo cell division after in vitro irradiation, as foreskin fibroblasts do.[40] These surviving fibroblasts that in vitro are capable of repopulating the tissue culture flask, in vivo might play a major role in stromal cell reconstitution after radiation-induced damage.

The hematopoietic supportive function of differentiated stromal layers is quite resistant to the effects of radiation, as shown by the coculture experiments. In vitro irradiation with doses in the range used in bone marrow transplantation does not impair the capacity of stromal layers to support prolonged granulomonopoiesis or their cellular composition. Although the curve of CFU-GM production may suggest that our coculture system is a declining one, detailed analysis of CFU-GM kinetics clearly shows that it supports active hematopoiesis. The culture flask can be viewed as a single compartment in which several phenomena occur simultaneously. In the absence of an adherent layer, there is no significant input of CFU-GM from an earlier compartment, whereas in the presence of an adherent layer, there is an active supply of CFU-GM by early progenitors. This explains the slower decline of CFU-GM frequency in cultures with adherent layers (six to eight *v* two to three weeks). The curves decline over time in both situations because the cell output exceeds the cell input. Cell output includes death, weekly demipopulation, and stem cell loss by differentiation. Data supporting active CFU-GM generation in this coculture system include (a) the six-week CFU-GM cumulative number in this study showing a higher CFU-GM content than in the recharge inoculum and (b) emergence of CFU-GM from earlier progenitors in cocultures of marrow cells depleted of CFU-GM by chemotherapeutic agents[41] or anti-Ia antibodies.[42]

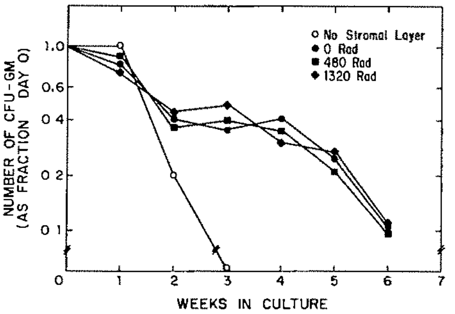

Fig 5. CFU-GM production in cocultures of irradiated confluent stromal layers with autologous hematopoietic cells. Four- to 6-week-old confluent stromal layers derived from nonirradiated marrows were irradiated and then cocultured with 6 × 10^6 freshly isolated autologous NAL marrow cells. CFU-GM production was monitored weekly. Results shown represent the mean of five experiments. Note that doses of irradiation up to 1,320 rad did not impair the hematopoietic supportive function of these layers.

The coculture system in which hematopoietic cells (donor cells) are added to established stromal layers (host stroma) reproduces in vitro the bone marrow transplant situation. Our in vitro observations parallel what occurs in human marrow transplantation. That donor hematopoietic cells engraft after irradiation of recipients with 1,000 rad[43] suggests that the hematopoietic supportive function of marrow stromal cells remains intact. Further evidence for the radioresistance of the hematopoietic supportive function has been provided by repopulation studies[14] showing active hematopoiesis in marrows that had been locally irradiated with doses up to 3,000 rad. It is of interest that in the murine system, a lower radioresistance of stromal function has been noted by some investigators,[22,23,44] while others observed a relatively radioresistant stromal function.[45] Whether these results represent different radiobiological properties or are due to the assay systems remains to be clarified. Our in vitro results in humans correlate well with in vivo observations showing an intact hematopoietic supportive function after irradiation with doses used for marrow transplantation.

Although it is well accepted that an intact marrow stroma is an absolute requirement for donor stem cell engraftment and sustained hematopoietic reconstitution, the role of host or donor stromal cells in this process in marrow recipients myeloablated with total body irradiation has not been clearly defined. This is in part due to the controversy regarding the origin and transplantability of the marrow stromal cells post marrow transplantation.[46-49] In any case, it appears that the host stroma plays a critical role in the early posttransplant period. Assuming that the marrow stroma is transplantable, it is unlikely that the donor stroma would both engraft and at the same time provide the conditions needed for donor stem cell "homing." Therefore, the host stroma has to provide the microenvironmental conditions until donor stromal cells populate the marrow and resume their hematopoietic supportive function. If nontransplantable, then the host stroma must maintain its normal hematopoietic supportive function and proliferative capacity to ensure, first, donor hematopoietic engraftment and, second, stromal reconstitution. Extrapolating our in vitro data to the marrow transplant situation, we can conclude that in the early stages of the posttransplant period, irradiated host mature stromal cells can provide the microenvironmental conditions needed for the seeding of donor hematopoietic stem cells and that host stromal cells retaining their proliferative capacity after irradiation can reconstitute the marrow stromal population.

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Restricted Expression of a New Acute Myelogenous Leukemia–Associated Antigen (NHL-30.5) on Normal Hemopoietic Progenitor Cells

By David S. Askew, Allen C. Eaves, Connie J. Eaves, and Fumio Takei

We have previously reported the isolation of a monoclonal antibody (NHL-30.5) that reacts with an antigen expressed on a substantial proportion of marrow and blood cells of most patients with newly diagnosed or relapsing acute myeloid leukemia. This antigen is also found on several cell lines derived from myeloid malignancies of human origin. It is not present on mature hemopoietic cells or on the majority of differentiating bone marrow cells. In order to determine whether the NHL-30.5 antigen may, nevertheless, be expressed on low-frequency primitive normal hemopoietic cells, not detected in standard antibody screening procedures, its expression was studied on clonogenic erythropoietic and granulopoietic cells. Light-density (<1.077 g/mL) suspensions of normal or chronic myelogenous leukemia bone marrow and peripheral blood cells were stained with NHL-30.5 and fluorescein isothiocyanate labeled second antibody and then sorted into two fractions using the fluorescence-activated cell sorter. The first contained the top 5% of cells with the highest fluorescence intensity. The remainder were collected in the second fraction. Colony assays of both fractions showed the first to be enriched in CFU-E, BFU-E, and CFU-C content (fourfold to 17-fold). The second fraction was correspondingly depleted of these progenitors. These findings reveal NHL-30.5 antigen expression to be a transient event during normal hemopoiesis that characterizes primitive hemopoietic cells on several pathways. Subsequent experiments showed that the presence of up to 10 μg/mL of purified NHL-30.5 antibody in colony assay cultures neither inhibited nor stimulated colony formation. Marrow fibroblasts (subcultured marrow adherent cells) were NHL-30.5 negative. Immunoprecipitation studies showed that the antigen detected by NHL-30.5 is clearly distinct from that identified by My-10, another monoclonal antibody that has previously shown some similarities to NHL-30.5. It thus appears that the NHL-30.5 antibody reacts with a new myeloid differentiation antigen of as yet unidentified function that is normally restricted in its expression to early stages of hemopoiesis.

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A VARIETY of circulating blood cell types are recognized by their unique morphological, antigenic, and functional properties. Current evidence indicates that the differentiation of these cells begins with the activation in primitive pluripotent progenitors of unique programs of gene expression that may take many cell generations to be completed.[1] The execution of such programs leads finally to the acquisition of specialized properties, although preliminary changes, including changes in responsiveness to various extrinsic growth factors, are also thought to occur. Some changes have been correlated with the loss of differentiative and proliferative potential that distinguish different populations of committed and pluripotent hemopoietic progenitors in clonogenic assays.[2] Nevertheless, there are relatively few examples of well-characterized intracellular or surface components that appear to be consistently altered during the earliest stages of hemopoietic progenitor differentiation.

One strategy for identifying relevant gene products is to prepare monoclonal antibodies against antigens that appear to be unique to leukemic cells.[3–9] This approach is based on the assumption that leukemic blasts may express surface antigens normally restricted to rare subpopulations of primitive hemopoietic cells. To enhance the selection of antibodies against such antigens, we raised monoclonals against HL-60 cell surface antigens and identified one (NHL-30.5) whose ability to bind to HL-60 cells was lost when these were induced to differentiate.[9,10] Subsequent studies showed that this monoclonal also reacted with a detectable proportion of blood and marrow cells from more than 80% of patients with newly diagnosed or relapsing acute myeloid leukemia (AML).[10] In contrast, mature blood cells, including granulocytes, lymphocytes, monocytes, and platelets, from either normal individuals or patients with chronic myelogenous leukemia (CML), were all found to be negative.[9] Similarly, by fluorescence-activated cell sorting (FACS) analysis, a detectable (>2%) positive population could not initially be demonstrated in buffy coat preparations of normal or CML marrow aspirates.[9]

Subsequent studies with light-density peripheral blood preparations from patients with CML revealed a small but clearly detectable NHL-30.5–positive population in some cases. A preliminary study with more highly purified populations of CML clonogenic granulopoietic progenitor cells also indicated that these reacted with NHL-30.5 (P.M. Lansdorp, personal communication, September, 1984). In this study, we have undertaken a more extensive evaluation of NHL-30.5 antigen expression on clonogenic progenitors, looked for a role of this antigen in progenitor cell proliferation and differentiation, and compared the antigens immunoprecipitated by NHL-30.5 and My-10.[7]

MATERIALS AND METHODS

Monoclonal antibodies The production of the NHL-30.5 monoclonal antibody, and its reactivity with normal and leukemic hemopoietic cells, has been described.[9] Hybridoma supernatant of the My-10 monoclonal antibody[7] was kindly provided by Dr Curt Civin (The Johns Hopkins Medical School, Baltimore).

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Preparation of human cells Heparinized peripheral blood was obtained from patients with CML in established chronic phase. Normal marrow specimens were obtained from transplant donors or patients with malignancies that did not involve the marrow Informed consent was obtained in each case. The light-density fraction of all specimens was isolated on a 1.077 g/mL solution of Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Md), according to the recommendations of the supplier.

FACS analysis. Cells were stained for FACS analysis as previously described[9] and analyzed on a FACS 440 equipped with a logarithmic amplifier (Becton Dickinson, Sunnyvale, Calif). Control cells were reacted with fluorescein isothiocyanate (FITC)-conjugated antibody only. The FACS was routinely standardized, using glutaraldehyde-fixed chicken red blood cells and fluorescent microspheres. Debris was gated out on the basis of light scatter measurements. With this set up, we routinely detect B cells in the peripheral blood (5% to 15% population). In order to determine the progenitor recovery, the number of cells in each sorted fraction (from microscopic counts) was multiplied by the frequency of progenitors in the same fraction and divided by the number of progenitors present in the cell suspension before sorting. Calculated progenitor recoveries varied from 19% to 171%, with an overall mean of 78% ± 6%

Immunoprecipitation The iodogen procedure was used to label KG-1 cell surfaces, and antigens were precipitated as previously described [9] Immunoprecipitates were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Assays for clonogenic myeloid progenitors. Cells were plated in standard 0.8% methylcellulose culture medium containing 30% fetal calf serum (FCS), 1% deionized bovine serum albumin, 10^{-4} mol/L 2-mercaptoethanol, 3 U/mL of human urinary erythropoietin (100 to 1,000 U/mg),[11] and 9% human leukocyte conditioned medium.[12] Normal unsorted bone marrow cells were plated at 1×10^5 cells per milliliter and peripheral blood at 4×10^5 cells per milliliter. Unsorted CML cells and all sorted cells were also plated at multiple lower cell concentrations to ensure obtaining a condition in which colony numbers were sufficient but not excessive. All evaluable cultures were scored and the data obtained consistent with a linear cell dose-colony yield relationship. Colonies derived from CFU-E (one to two clusters of erythroblasts) and mature BFU-E (three to eight clusters of erythroblasts) were counted on day 10 and from primitive BFU-E (more than eight clusters of erythroblasts) and CFU-C (more than 20 granulocytes and/or macrophages), on day 18, according to established criteria.[1]

For progenitor cell inhibition/stimulation studies, 1 to 10 μg/mL of purified NHL-30.5 monoclonal antibody was included in the assay medium. Assays both with and without erythropoietin and leukocyte conditioned medium were used in these experiments.

RESULTS

FACS analysis. Because the peripheral blood of patients with CML typically contains elevated numbers of progenitors,[13-15] CML blood was first used to investigate the expression of the NHL-30.5 antigen on these primitive cell types. Cells from four patients were studied. Depending on the patient, 1% to 11% of the Ficoll-Hypaque–separated CML cells were found to be NHL-30.5 positive. In the cases where <5% of the cells were positive, the sort gates were adjusted so that cells with the highest fluorescence intensity, constituting 5% of the total population, were sorted into the positive fraction and cells with the lowest fluorescence (the remaining 95%) were sorted into the negative fraction. Aliquots from both the positive and the negative fractions were then plated in standard methylcellulose assays. Table 1 shows the numbers of CFU-C, BFU-E, and CFU-E colonies per 10^5 cells measured in these experiments. In the positive fraction, granulocyte–macrophage progenitors were enriched an average of fourfold, while BFU-E and CFU-E were enriched sixfold and sevenfold, respectively. Both the enrichment of progenitors in the positive fraction and their corresponding depletion from the negative fraction were significant ($P < .05$, Table 1).

Ficoll-Hypaque–separated normal bone marrow and peripheral blood cells were than stained, sorted, and assayed

Table 1. Number of Progenitors per 10^5 Cells in Sorted Fractions From CML Peripheral Blood

Patient No.	1	2	3	4	Mean of Four Experiments*	Recovery of Progenitors (%)
CFU-C						
Control	207.0	121.0	184 0	1535	290 (164–512)	
(+) Fraction	1,092.0	414.0	1,120 0	3750	1,174 (748–1,843)	41 ± 7
(−) Fraction	17.0	14.0	34.0	205	36 (20-67)	12 ± 2
CFU-E						
Control	262.0	443.0	46.0	1055	275 (142–530)	
(+) Fraction	2,530.0	3,200.0	360.0	5610	2,012 (1,107–3,654)	73 ± 9
(−) Fraction	83.0	132.0	19.0	173	78 (48–127)	27 ± 5
BFU-E						
Control	80.0	167.0	39.0	1780	175 (76–401)	
(+) Fraction	1,050.0	979.0	290.0	3120	982 (605–1,596)	68 ± 25
(−) Fraction	30.0	79.0	20.0	263	60 (34–106)	34 ± 8
CFU-GEMM						
Control	1.7	5.3	0.0	0.0		
(+) Fraction	164.4	6.5	2.5	0.5		
(−) Fraction	0.0	1.2	0.0	0.0		

Three fractions were obtained: an NHL-30.5–positive fraction, an NHL-30.5–negative fraction, and an unsorted fraction Enrichment of all progenitors in the positive fraction and corresponding depletion from the negative fraction was significant when compared with the control in a one-tailed paired-sample t test ($P < .05$). Percentage of recovery was calculated as described in Materials and Methods

*Geometric mean (range defined by ± 1 SEM) in a similar fashion. Although a distinct NHL-30.5–positive population was not observed in either normal marrow or peripheral blood, the sort gates were adjusted in each case so that cells with the highest fluorescence intensity, again constituting 5% of the total population, were sorted into the positive fraction. Table 2 shows the results from experiments with six different normal marrow specimens. CFU-C were consistently and significantly ($P < .05$) enriched in the NHL-30.5–positive fraction (on average, approximately 17-fold). Significant ($P < .05$) enrichment of BFU-E and CFU-E was also demonstrated, with corresponding depletion of these progenitors from the negative fractions. Although the anomalous behavior of erythroid progenitors from bone marrow No. 3 (Table 2) suggests the existence of NHL-30.5–negative erythroid progenitors in this patient, the possibility that this result was an artifact cannot be ruled out. When the light-density mononuclear fraction of normal peripheral blood was sorted in a similar manner, the positive fraction again showed significant ($P < .05$) enrichment of all progenitor types: tenfold for CFU-C, fivefold for CFU-E, and fivefold for BFU-E (data not shown). Small numbers of CFU-GEMM (progenitors of mixed granulocyte–erythroid–macrophage colonies) were observed in some experiments where the general trend suggested a similar staining behavior to that of other clonogenic progenitors.

In some experiments, a control sample of cells was stained with the second antibody alone and the top 5% again sorted and assayed for progenitors. In such experiments, no enrichment of any progenitor type was obtained, thus providing direct evidence that the progenitor enrichment observed with NHL-30.5 staining was due to selective reactivity with this antibody.

To test whether marrow fibroblasts and other minor adherent cell components of normal marrow aspirates express NHL-30.5, marrow adherent layers were established in 20% FCS in alpha medium by seeding 1 to 2 × 10^7 cells into 60-mm tissue culture dishes and maintaining them at 37 °C with weekly feeding. After one or two subcultures, cells were allowed to reach confluence and then collagenased[16] before staining for FACS analysis. Such cell suspensions contained no detectable (<1%) NHL-30.5–positive cells (three separate marrows tested), whereas concurrently analyzed and collagenase-treated HL-60 cells were 40% positive.

Functional studies of NHL-30.5. Additional experiments were undertaken to investigate whether the NHL-30.5 antigen might be involved in progenitor proliferation or differentiation responses. To test these possibilities, purified NHL-30.5 antibody was incorporated into the assay medium (up to 10 μg/mL). This, however, had no detectable inhibitory or stimulatory effect on colony formation by normal peripheral blood or marrow progenitors by comparison with control cultures plated both with and without stimulators (erythropoietin and leukocyte conditioned medium) (three experiments, data not shown). Addition of an equivalent concentration of mouse Ig (Sigma Chemical Co, St Louis) was also without effect.

Immunoprecipitation. Because the pattern of reactivity of NHL-30.5 is most reminiscent of that observed with the My-10 monoclonal antibody,[7] both antigens were compared directly by immunoprecipitation of surface components of KG-1 cells. The KG-1 line was selected because the My-10 antigen is absent from HL-60 cells.[7] The proteins immunoprecipitated with these two antibodies were clearly different in their mobility in SDS-PAGE (Fig 1). The NHL-30.5 antigen was shown to have a molecular weight (mol wt) of 180,000 under both reducing and nonreducing conditions, as previously demonstrated for HL-60 cells.[9] My-10 precipi-

Table 2. Number of Progenitors per 10^5 Cells in Sorted Fractions From Normal Bone Marrow

Patient No	1	2	3	4	5	6	Mean of Six Experiments*	Recovery of Progenitors (%)
CFU-C								
Control	103	93.0	102.0	247.0	127.0	197.0	135 (114–159)	
(+) Fraction	2,267	2,054.0	1,630.0	2,640.0	3,308.0	2,350.0	2,320 (2,105–2,556)	72 ± 14
(−) Fraction	20	15.0	72.0	15.0	69.0	19.0	28 (20–37)	27 ± 13
CFU-E								
Control	221	182.0	225.0	278.0	190.0	67.0	178 (145–219)	
(+) Fraction	2,856	3,243.0	340.0	3,460.0	3,487.0	1,220.0	1,896 (1,296–2,772)	42 ± 16
(−) Fraction	144	21.0	229.0	28.0	268.0	17.0	67 (40–112)	45 ± 21
BFU-E								
Control	55	65.0	93.0	171.0	27.0	197.0	82 (61–111)	
(+) Fraction	250	946.0	75.0	1,530.0	450.0	2,350.0	554 (330–930)	29 ± 13
(−) Fraction	34	13.0	105.0	42.0	64.0	19.0	37 (27–50)	52 ± 20
CFU-GEMM								
Control	1	1.5	0.5	0.4	0	0		
(+) Fraction	0	13.5	0.0	0.0	0	0		
(−) Fraction	0	0.4	0.0	0.0	0	0		

There was significant enrichment of all progenitors in the positive fraction when compared with the control fraction in a one-tailed paired-sample *t* test ($P < .05$). Depletion from the negative fraction was significant for both CFU-C and CFU-E but was not significant for BFU-E (experiments 3 and 5 were not depleted of BFU-E in the negative fraction). Percentage of recovery was calculated as described in Materials and Methods.

*Geometric mean (range defined by ±1 SEM)

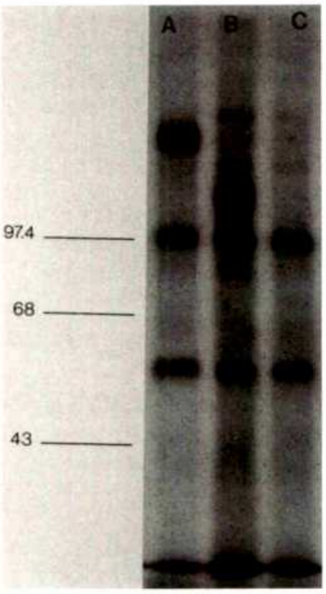

Fig 1. A comparison of the NHL-30.5 and My-10 antigens by immunoprecipitation. KG-1 cells were surface labeled with ^{125}I, and antigens were immunoprecipitated from the cell lysate, using the NHL-30.5 or My-10 monoclonal antibodies. Analysis was carried out on SDS-PAGE (7.5%) under reducing conditions. Lane A: NHL-30.5 monoclonal antibody; lane B: My-10 monoclonal antibody; lane C: negative control, an unrelated antibody raised against mouse lymphocytes.

tated a smaller moiety, with a mol wt of approximately 115,000 from the same cells.

DISCUSSION

The purpose of the study was to investigate the possible distribution of NHL-30.5 on uncommon but normal marrow elements whose presence would have escaped detection in standard binding studies or FACS analyses.[9,10] Such studies had shown that purified populations of lymphocytes, monocytes, granulocytes, erythrocytes, and platelets from normal individuals did not react with this antibody and that normal bone marrow buffy coat preparations contained less than 2% NHL-30.5–positive cells.[9] In the experiments described in this report, the light-density fraction of all specimens was obtained by centrifugation on Ficoll-Hypaque (density, 1.077 g/mL). This appeared to increase the number of NHL-30.5–positive cells by a few percent. As a result, a small but distinct (>5%) positive population could sometimes be demonstrated in the peripheral blood of CML patients in chronic phase. However, this was not the case for either normal marrow or peripheral blood samples, in which NHL-30.5–positive cells failed to reach the 5% level. Nevertheless, when cells with the highest fluorescence intensity (constituting 5% of the total population) were sorted and assayed, a significant and selective enrichment of clonogenic progenitors, including CFU-E and BFU-E as well as CFU-C, was obtained. These findings suggest that expression of NHL-30.5 is part of an early stage of hemopoietic cell differentiation that persists even after lineage restriction. In contrast to the positive results obtained with clonogenic progenitors, assessment of adherent marrow cell populations that are capable of regulating progenitor turnover in vitro (unpublished observations, June 1985) showed these to be NHL-30.5 negative.

Few monoclonal antibody reagents are currently available that identify antigens restricted in their expression to early stages of myeloid cell differentiation.[3,7,8] The My-10 monoclonal antibody[7] has a pattern of reactivity that shows some similarity to that of NHL-30.5 in that both react with immature myeloid populations and some leukemic blasts, but not with differentiated myeloid cells. The present study establishes that the antigen detected by My-10 is electrophoretically distinct from that detected by NHL-30.5. The former has an approximate mol wt of 115,000 in contrast to 180,000, the mol wt of the NHL-30.5 antigen. Consistent with this difference between My-10 and NHL-30.5 is the finding that NHL-30.5 reacts with cells from most (80%) patients with AML,[10] whereas My-10 appears to react with cells only from a minority (28%) of AML patients.[7]

The stage of normal hemopoietic cell differentiation at which NHL-30.5 first appears remains to be determined. Our limited observations to date suggest that clonogenic pluripotent progenitors (CFU-GEMM), like their various lineage-restricted but clonogenic progeny, are NHL-30.5 positive. However, even CFU-GEMM are not believed to represent stem cells with long-term repopulating potential. In standard short-term assays, most CFU-GEMM display limited self-renewal capacity[17-19] and thus differ from high self-renewal pluripotent cells that have recently been shown to be initially refractory to growth stimuli in vitro.[20,21] It was recently reported that the lineage-restricted progenitors observed in 4 to 6-week-old long-term marrow cultures may be derived from a population of cells that differ in HLA-DR expression from CFU-GEMM as well as CFU-C and BFU-E.[22] Experiments are currently under way to determine whether such differences also extend to NHL-30.5 expression.

The normal functional role of NHL-30.5 is also unknown. Because most clonogenic progenitors in normal peripheral blood are not actively cycling, in contrast to those in CML blood or all but the most primitive classes present in normal bone marrow,[23-27] it seems unlikely that regulation of NHL-30.5 expression is directly related to cell cycle status. Tests to evaluate whether NHL-30.5 binding could inhibit or stimulate normal progenitor proliferation and differentiation also showed no effect. Although this observation may reflect the particular epitope recognized by the NHL-30.5 antibody rather than the physiologic role of the antigen of which it is a part, surface functions other than those involved in colony formation need to be examined. The fact that deregulation of expression of the NHL-30.5 antigen appears to be a common event in AML may provide some clues in this regard.

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Activation of Human Granulocytes by Arachidonic Acid: Its Use and Limitations for Investigating Granulocyte Functions

By Harvey J. Cohen, Margaret E. Chovaniec, Kazuhiko Takahashi, and John C. Whitin

The effects of arachidonic acid on human granulocytes (polymorphonuclear neutrophil leukocytes, PMN) were examined with respect to early events associated with activation of the superoxide (O_2^-)-generating system and its reactivation with other stimuli after the addition of fatty acid–free bovine serum albumin. As with other stimuli, O_2^- production occurred after a lag that was dependent on the amount of arachidonic acid used. Although the lag, rate, and extent of O_2^- production were dose dependent, at all concentrations used, the duration of O_2^- production was four to six minutes. As previously described, when fatty acid–free albumin was added one minute after stimulation of PMN by arachidonic acid, O_2^- production ceased immediately. The O_2^--generating system could then be reactivated by the addition of either phorbol myristate acetate (PMA), concanavalin A, or serum-treated zymosan. In previously activated cells, the lag time for reactivation was shorter and the rate of O_2^- production greater with PMA. PMN membrane potential was depolarized by arachidonic acid. This depolarization was not reversed with the addition of albumin. PMN cytoplasts were also capable of reversible activation by arachidonic acid in a manner identical to whole cells. Divalent cations were found to be necessary for the activation of PMN by arachidonic acid. In the absence of calcium, arachidonic acid caused rapid lysis of the cells, as manifested by the release of lactic acid dehydrogenase. We were unable to measure later events in PMN stimulated by arachidonic acid since even in the presence of divalent cations lactic acid dehydrogenase was released from the cells after five minutes of incubation. We conclude that, in addition to its ability to activate PMN, arachidonic acid is toxic to the cells and cannot be used to study late events in granulocyte activity, and, in the absence of calcium, it may be difficult to interpret its action as a stimulant of the oxidase.

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THE SUPEROXIDE (O_2^-)-generating system in human granulocytes (polymorphonuclear neutrophil leukocytes, PMN) appears to be a multicomponent enzyme complex consisting of one or more of the following components: flavoprotein,[1,2] cytochrome b,[3,4] ubiquinone,[5] and sulfhydryl groups.[6] The subcellular localization and translocation of these components has recently received much attention.[7–10] Resting PMN produce little[11] if any[12] O_2^-. Upon interaction with appropriate soluble and particulate stimuli, a complex series of events occur that include changes in membrane potential,[13,14] an increase in intracellular ionized calcium,[15,16] changes in cyclic nucleotides,[17] and the use of adenosine triphosphate (ATP).[18] These events accompany and some may be causally related to the activation of an NADPH oxidase, which results in the production of O_2^- and the other aspects of the respiratory burst.

The nature of the activation process is still largely unknown. Events such as phosphorylation,[19] proteolysis,[20] and protein translocation[9] have been postulated to be responsible for this activation. Curnutte et al have previously shown that O_2^- production by fluoride-treated PMN ceases when the cells are washed. O_2^- production could be reinstated with additional fluoride.[21] We have also shown that the activation of the O_2^--generating system by concanavalin A (Con A) can be reversibly inactivated with the addition of either α-methyl mannoside (α-MM) or tosylphenylalanylchloromethylketone.[22] Korchak et al have recently shown reversible activation by formylmethionylleucylphenylalanine (FMLP).[23] In this manuscript, we extend these observations on reversible activation using arachidonic acid as an activator of the PMN O_2^- generating system. Studies by other investigators have shown that arachidonic acid stimulation can be reversed with fatty acid–free bovine serum albumin.[24–25] We have investigated the nature of this reversible activation in both intact PMN and enucleated PMN or cytoplasts.[26] The similar results obtained in both systems eliminates certain processes in both the inactivation and reactivation of the PMN O_2^--generating system. We have, however, also found that archidonic acid produces irreversible damage to PMN that limits its use in investigating the latter aspects of the respiratory burst.

MATERIALS AND METHODS

Cytochrome c (type VI), superoxide dismutase, arachidonic acid (99% pure), cytochalasin B, Con A, α-MM, fatty acid–free bovine serum albumin, FMLP, *Micrococcus lysodeikticus*, NADPH, NADH, pyruvate, *p*-nitrophenyl phosphate, and Triton X-100 were purchased from Sigma Chemical Co, St Louis; Dextran T500, Ficoll-Paque and Ficoll 70 from Pharmacia Fine Chemicals, Piscataway, NJ; phorbol myristate acetate (PMA) from Consolidated Midland Chemical Co, Brewster, NY, 3,3′-dipropylthiodicarbocyanine (diSC_3-5) from Molecular Probes, Junction City, Ore; and zymosan from ICN Pharmaceuticals, Plainview, NY These and all other reagents were obtained at the highest level of purity and used without further purification. All surfaces in contact with cells were plastic or siliconized glass except for spectrophotometer cuvettes.

Preparation of PMN. Peripheral blood was collected in acid-citrate-dextrose and PMN were purified by dextran and Ficoll-Paque sedimentation as described previously.[27] The cells (95% to 98% PMN) were washed and suspended in phosphate-buffered saline (PBS)[28] at 2 to 5 × 10^7/mL All cells and enzymes were maintained on ice until used. Arachidonic acid was stored under vacuum in sealed vials at a concentration of 10 mg/mL in ethanol and was diluted to 2.5 mg/mL with water and clarified with 0.1 N NaOH prior to use. High-performance liquid chromatography (HPLC) analysis of the stored arachidonic acid demonstrated that there were less than 1% oxidation products of arachidonic acid The

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ability of arachidonic acid to stimulate PMN did not vary during the course of the day's experiments.[24] PMA,[29] Con A,[22] and serum-treated zymosan (STZ)[30] were prepared as previously described.

Preparation of cytoplasts. Neutrophilic cytoplasts were prepared as previously described.[26] PMN were suspended in a 12.5% (wt/vol) Ficoll 70 solution containing 10 μg/mL of cytochalasin B and heated for five minutes at 37 °C. The suspension was layered on a prewarmed, discontinuous gradient of 16% (wt/vol) and 25% (wt/vol) Ficoll 70. Cytochalasin B (10 μg/mL) was present through the gradient. The gradients were centrifuged for 30 minutes at 81,000 g (middle of the tubes) at 33 °C. After centrifugation, the cytoplasts were harvested at the interface of the 12.5% and 16% Ficoll solutions. The cytoplast fractions were washed five times with PBS by centrifugation for ten minutes at 800 g at 20 °C. As a control, granulocytes were incubated with 12.5% Ficoll 70 and 10 μg/mL cytochalasin B for 30 minutes at 33 °C and washed five times with PBS. Alkaline phosphatase as a marker of the plasma membrane was measured by the method of Roos et al.[26]

Assay for O_2^- production Continuous spectrophotometric measurements of O_2^--dependent cytochrome c reduction was performed as previously described[29] in a Varian DMS 100 double-beam spectrophotometer with a water-jacketed cell compartment set at 37 °C. The sample cuvette contained ferricytochrome c (100 nmol), PMN (1 to 2.5 × 10^6), or equivalent amounts of cytoplasts and the appropriate stimulant In addition, the reference cuvette contained superoxide dismutase (20 μg). Lag times were recorded as previously described,[12] with the chart recorder started at the time of the addition of the stimulant to the sample cuvette.

Membrane potential depolarization. PMN membrane potential changes induced by arachidonic acid could not be continuously monitored using fluorescent probes as we had previously done in studies of PMA-,[13] STZ-,[30] and Con A-[31] induced depolarization. Addition of arachidonic acid to cell-free carbocyanine dye in buffer resulted in complete quenching of its fluorescence. This effect was reversed by the subsequent addition of albumin. The quenching problem was circumvented by exploiting the ability of albumin to rapidly bind arachidonic acid. PMN were incubated with arachidonic acid in microcentrifuge tubes for varying lengths of time Albumin was then added and the incubation continued for a total elapsed time of two minutes for all samples. At that time, the samples were centrifuged for 5 seconds, the supernatant discarded, and the cells resuspended in Krebs Ringer Phosphate ([KRP] PBS plus 1 mmol/L $CaCl_2$ and 1.2 mmol/L $MgSo_4$). The membrane potential of these cells was then assessed by incubating them at 2 × 10^6/mL with 2 μmol/L $diSC_3$-5 for seven minutes and measuring the fluorescence in a Perkin Elmer Model 650-10S spectrofluorometer

Degranulation. Cell suspensions (1 × 10^7/mL) were incubated with arachidonic acid at various concentrations, Con A (100 μg/mL), or PMA (1 μg/mL) for the times noted. For the arachidonic acid-treated cells, albumin (1.4 mg/mL) was added at various times. In each instance, the cells were centrifuged in a microcentrifuge for one minute and the supernatants assayed for lysozyme and lactate dehydrogenase (LDH) activity. Lysozyme activity was measured at 450 nm by following the lysis of *Micrococcus lysodeikticus* as previously described.[32] LDH activity was measured by the continuous spectrophotometric method of Beutler.[33] Whole cells were lysed with 0.05% Triton X-100 and assayed for total enzyme content using the equivalent of 2 to 4 × 10^5 PMN per assay.

RESULTS

Arachidonic acid–stimulated superoxide production. Previous investigators have shown that addition of arachidonic acid to PMN stimulates O_2^- production.[24] It has been stated that this superoxide production occurs without a lag time following the addition of arachidonic acid.[25] As can be seen in Fig 1, there is a definite lag time prior to complete activation of the O_2^--generating system by arachidonic acid. We have found that this lag time is 15 to 30 seconds at 37 °C. The duration of superoxide production by arachidonic acid–stimulated PMN is quite short, terminating in three to five minutes. O_2^- production in these samples could not be restored by the addition of either more PMN or more arachidonic acid. The rapid termination of O_2^- production was not due to the release of toxic substances from the PMN since the addition of both fresh PMN and arachidonic acid to the samples resulted in a resumption of O_2^- production after the appropriate lag. This was true even when the fresh PMN were allowed to incubate for up to six minutes in the assay cuvettes containing the original cells and arachidonic acid prior to the second addition of arachidonic acid. As can also be seen in Fig 1, the duration of O_2^- production by arachidonic acid is unaffected by the rate or extent of O_2^- production. At a lower concentration of arachidonic acid both the rate and extent of O_2^- production are diminished, but the duration of the burst is the same as for the higher concentration of arachidonic acid. Neither the rate nor the duration of O_2^- production by arachidonic acid is affected by the addition of cytochalasin B. The lag time for arachidonic acid is shorter than for most stimuli but is still dependent, as is the rate, on the concentration of arachidonic acid used to activate the cells, as shown in Fig 2.

Reversibility of arachidonic acid–induced superoxide production. It has previously been shown that the addition of albumin to arachidonic acid–stimulated PMN resulted in an immediate cessation of O_2^- production.[24] The addition of more arachidonic acid then resulted in a reinstitution of O_2^- production. O_2^- production can be reinstituted in PMN previously treated with arachidonic acid followed by albumin after the addition of either PMA, STZ, or Con A. Figure 3 demonstrates that the maximum attainable rate of O_2^- production induced by PMA is the same whether or not the cells were previously activated with arachidonic acid. At lower, nonsaturating concentration of PMA, the rate of O_2^- production induced by PMA is greater and the lag times are shorter when the PMN had previously been stimulated with arachidonic acid. These results are similar to our previous

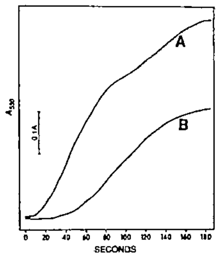

Fig 1. O_2^- production by arachidonic acid–stimulated PMN. Superoxide dismutase–inhibitable O_2^- production by 1 × 10^6 PMN in KRP was monitored (as the change in absorbance) at 550 nm of cytochrome c in a double-beam spectrophotometer as described in the Methods section in the presence of (A) 25 μg or (B) 6.3 μg of arachidonic acid added at zero time. Although the lag time, rate, and extent of O_2^- production were different, the duration of O_2^- production was the same at each concentration.

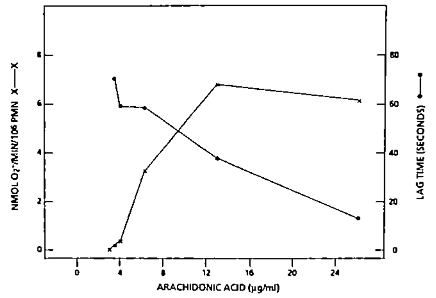

Fig 2. Effect of arachidonic acid concentration on PMN O_2^- production. Arachidonic acid, at the concentrations noted, was added to PMN, and the initial linear rates of O_2^- production (×) and lag times for O_2^- production (●) were measured at 37 °C. The results are the average of duplicate determinations.

findings in Con A– and α-MM–treated PMN and indicate that previously activated PMN are primed for reactivation with a shorter lag and an increased sensitivity to the second stimulus.

Membrane potential depolarization by arachidonic acid. The membrane potential of cells treated with arachidonic acid and albumin was assessed using the fluorescent dye diSC$_3$-5 as described in the Methods section. The results, as shown in Fig 4, indicate that depolarization appears to begin within 5 seconds after interaction of the cells with arachidonic acid. The maximum extent of depolarization appears to occur after 30 seconds. This correlates with the time necessary for activation of the O_2^--generating system by arachidonic acid. As has previously been shown for α-MM treatment of Con A–stimulated PMN,[31] inactivation of the O_2^--generating system by albumin does not reverse the depolarization.

Time course for reactivation. As previously noted, the duration of O_2^- production induced by arachidonic acid is short and is not affected by treatment of PMN with cytochalasin B. We examined reactivation of arachidonic acid–treated PMN by PMA as a function of the time between the addition of arachidonic acid and albumin. The ability of PMA to reactivate PMN treated with arachidonic acid and albumin decreased with time, and the cells could not be reactivated by PMA when albumin was added five minutes after arachidonic acid (when arachidonic acid–induced O_2^- production had ceased). Similar results were obtained when STZ or Con A were used as second stimuli. In addition, even when albumin was added to PMN after only a short incubation with arachidonic acid, there was a time-dependent effect on the ability of these cells to be reactivated by PMA. When PMN were incubated with arachidonic acid for one minute prior to the addition of albumin, there was a time-dependent decrease in the ability of PMA to restimulate these cells, with the cells almost completely unresponsive after 30 minutes' incubation. This was in contrast to the results obtained with Con A as an initial stimulus followed by the addition of α-MM after two minutes. In this instance, PMA could restimulate these cells even after 30 minutes' incubation.

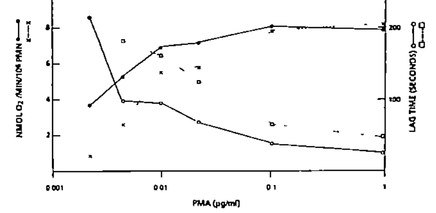

Fig 3. Effect of prior activation of PMN by arachidonic acid on responsiveness to PMA. PMN (1 × 10^6) were incubated in assay cuvettes with either arachidonic acid (12.5 μg) and albumin (1.4 mg) for two minutes prior to the addition of PMA (dashed lines) or arachidonic acid (12.5 μg) for one minute followed by albumin (1.4 mg) for an additional minute prior to the addition of PMA (solid lines). The rates of O_2^- production (×, ●) and the lag times (□, ○) are plotted as a function of the concentration of PMA used. The results are the average of duplicate determinations.

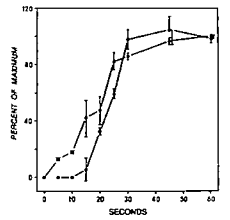

Fig 4. Comparison of arachidonic acid–induced membrane potential depolarization and rates of O_2^- production. Arachidonic acid (15.6 μg) was added to 2.5 × 10^6 PMN in 1.25 mL KRP, and at the indicated times albumin (1.75 mg) was added. The incubation was continued for a total of two minutes. The PMN were then rapidly centrifuged and resuspended in 0.25 mL KRP. These PMN (0.2 mL) were immediately added to 0.8 mL KRP and incubated with 2 μmol/L diSC$_3$-5. The steady-state level of fluorescence (a function of the membrane potential) was then recorded after seven minutes (●). An increase in fluorescence indicates membrane potential depolarization. In parallel experiments, the rate of arachidonic acid–stimulated O_2^- production was determined at the times noted (○) with the aid of a Varian DS-15 computer interfaced with the double-beam spectrophotometer. All results are given as the percentage of maximum values obtained at two minutes. The results are the mean and standard deviations of triplicate determinations.

Reversible activation of cytoplasts with arachidonic acid and Con A. PMN cytoplasts were prepared as previously described.[26] We compared the ability of arachidonic acid and Con A to activate these cytoplasts and the ability of second stimuli to reactivate these enucleated and granule-depleted cells. Figure 5 shows the ability of cytoplasts to respond to arachidonic acid and Con A. The rate of O_2^- production and the lag times when normalized to alkaline phosphatase content were similar for cytoplasts and PMN. The effects of albumin on arachidonic acid–induced O_2^- production and α-MM on Con A–induced O_2^- production by cytoplasts were identical to that seen for whole cells. Finally, reactivation of cytoplasts by a second stimulus, PMA, was the same for cytoplasts when compared with whole cells. Thus, granule-depleted cytoplasts are capable of undergoing reversible activation and reactivation in a manner similar to whole cells. In these experiments, cytochalasin B was added to the assay cuvettes to ensure maximal O_2^- production by Con A–stimulated cytoplasts. O_2^- production by arachidonic acid–stimulated cytoplasts did not require additional cytochalasin B.

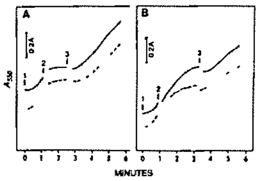

Fig 5. Reversible activation of cytoplasts by arachidonic acid and Con A. Cytoplasts were made as described in the Methods section. PMN (1 × 10^6) (solid lines) or the equivalent of 1 × 10^6 PMN as cytoplasts (dashed lines) were added to assay cuvettes in the presence of cytochalasin B (5 μg) and stimulated with (A) arachidonic acid (12.5 μg) or (B) Con A (100 μg). After one minute, albumin (1.4 mg) (A) or α-MM (25 μmol) (B) was added. After an additional one (A) or two (B) minutes, PMA (1.0 μg) was added to each sample. O_2^- production was monitored continuously after each addition.

Effects of divalent cations on arachidonic acid–dependent O_2^- production by PMN and cytoplasts. Most stimuli require external calcium for activation, but not for ongoing activity, of the PMN O_2^--generating system. We examined the calcium requirements for activation of PMN by arachidonic acid. Ethylene glycol tetra-acetic acid (EGTA), when added prior to the stimulant, prevented activation of PMN by arachidonic acid (data not shown), similar to what has previously been observed for most other stimuli. EGTA also prevented activation of the cytoplast O_2^--generating system by arachidonic acid (data not shown). Reactivation of PMN by second stimuli (with the exception of PMA) required the presence of divalent cations. When EGTA is added with albumin after stimulation of PMN with arachidonic acid prior to addition of either Con A or STZ, neither was capable of reactivating the O_2^--generating system. Reactivation of PMN by PMA was unaffected by the combined addition of EGTA and albumin. For Con A and STZ we have been able to demonstrate that EGTA has no effect on ongoing O_2^- production.[30,31] When EGTA was added to arachidonic acid–stimulated PMN, O_2^- production ceased immediately. However, these cells were incapable of responding to PMA, indicating that EGTA in the presence of arachidonic acid may have been toxic to the oxidase system. Similar results were obtained when arachidonic acid was added to PMN in the absence of divalent cations.

Effects of arachidonic acid on release of lysozyme and LDH. It has previously been reported that although O_2^- production by Con A– and FMLP-stimulated PMN can be reversed, degranulation continues.[23,31] We attempted to determine whether the same phenomenon occurred with arachidonic acid–treated cells. As can be also be seen in Fig 6, arachidonic acid stimulation of PMN resulted in the time-dependent release of lysozyme into the extracellular medium. As can also be seen in this figure, the addition of albumin at various times after stimulation by arachidonic acid did not completely prevent further enzyme release. We also compared LDH release by arachidonic acid–stimulated PMN with the release of lysozyme. Figure 6 also shows that after ten minutes significant amounts of LDH are also released and that at 30 minutes its release approaches that of lysozyme. The addition of albumin even one minute after arachidonic acid did not prevent further release of LDH. Thus, although it appears that lysozyme release occurs after O_2^- production ceases, we were unable to determine with certainty whether there was any specific release of granule contents following termination of O_2^- production with albumin. Lower concentrations of arachidonic acid (1 to 5 μg/mL) induced release of lysozyme without causing LDH release. However, these concentrations were not sufficiently high to activate the O_2^--generating system.

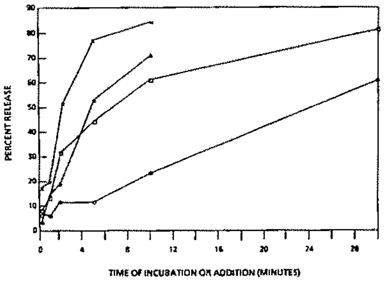

Fig 6. Arachidonic acid–stimulated lysozyme and LDH release and effect of albumin. Arachidonic acid (12.5 μg) was added to 2 × 10^6 PMN in KRP in a total volume of 1 mL. At the times noted, the cells were centrifuged for 10 seconds in a microfuge. The supernatant was then assayed for lysozyme (□) and LDH (○). To other PMN (2 × 10^6), arachidonic acid (12.5 μg) was added at zero time, and at the times noted, albumin (1.4 mg) was added. The incubations were continued for a total of 30 minutes, the cells centrifuged and the supernatant assayed for lysozyme (×) and LDH (△) content. The lysozyme and LDH content of PMN was determined and the results reported as the percentage of total cellular content of each enzyme released. The results are the average of duplicate determinations.

The effect of EGTA was further explored by examining lysozyme and LDH release at early (five-minute) and late (30-minute) times after the addition of arachidonic acid. As can be seen in Fig 7, for cells stimulated in KRP (A), lysozyme release after five minutes was greater than LDH release, with the two approximating each other after 30 minutes. In the presence of EGTA (B) or for cells stimulated in PBS (C), essentially maximal lysozyme and LDH release occurred within five minutes. Even when EGTA was added one minute after arachidonic acid (D), both lysozyme and LDH release occurred within five minutes. In other studies, we found that maximum LDH release occurred as early as two minutes after the addition of arachidonic acid to EGTA-treated cells. Figure 8 shows that it was the absence of Ca^{++} rather than Mg^{++} that resulted in this arachidonic acid–induced rapid lysis. No significant release of LDH occurred in PMN incubated in EGTA in the absence of a stimulus or in the presence of PMA (1 μg/mL) or Con A (100 μg/mL) and cytochalasin B (5 μg/mL). It therefore appears that in the absence of Ca^{++} arachidonic acid is rapidly toxic to PMN.

DISCUSSION

It has previously been reported that arachidonic acid activation of PMN results in the immediate onset of O_2^- production.[24,25] This was in contradistinction to all other stimuli, which induce O_2^- production after a lag time of 30 to 60 seconds. We examined this measure of activation at 37 °C in a continuous assay and found a short (15 to 30 second),

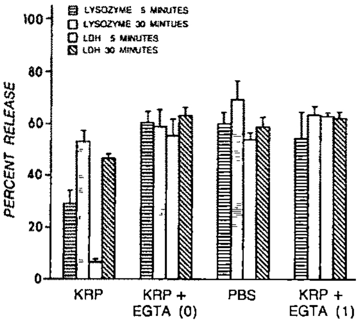

Fig 7. Effect of divalent cations on lysozyme and LDH release by arachidonic acid–stimulated PMN. As indicated on the x-axis, arachidonic acid (12.5 μg) was added to 2 × 10^6 PMN in KRP, KRP + 10 mmol/L EGTA (at time zero), or PBS. After five and 30 minutes, the cells were centrifuged and the supernatants assayed for lysozyme and LDH content. In the last condition noted on the x-axis, 10 mmol/L EGTA was added one minute after the arachidonic acid and the cells assayed for lysozyme and LDH release at five and 30 minutes. All results are expressed as the percentage of total cellular content of each enzyme released and are the mean and standard deviation of quadruplicate samples.

reproducible lag time for arachidonic acid that was dependent on concentration. This lag time appeared to correlate with the time necessary for maximal membrane potential depolarization. The uncertainty about this statement is the result of the assay necessary to measure the membrane potential change induced by arachidonic acid. If albumin is able to immediately halt depolarization as it is able to stop O_2^- production, then the times shown in Fig 5 are correct. If there is a delay prior to halting depolarization, then the maximal membrane potential change may occur at a later time then noted. Since there are demonstrable differences between the early time points, it is unlikely that there is a significant delay between the addition of albumin and the cessation of the membrane potential change. We have confirmed the reversibility of the activation of the O_2^--generating system by arachidonic acid with albumin and agree with Badway et al that it occurs within seconds.[24] Thus, although activation requires a lag time, inactivation of arachidonic acid–treated PMN occurs within 5 seconds (the time necessary to obtain the first measurements). This rate of inactivation is faster than that occurring when α-MM reverses the activation of Con A–induced O_2^- production[22] but is similar to that seen for the reversal of FMLP activation.[23] In all instances, reactivation with other stimuli can occur with a shorter, but still demonstrable, lag time.

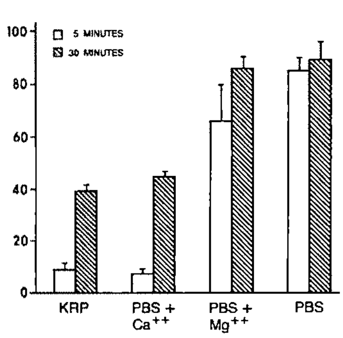

Fig 8. Effect of calcium or magnesium on LDH release by arachidonic acid–stimulated PMN. As indicated on the x-axis, arachidonic acid (12.5 μg) was added to 2 × 10^6 PMN KRP, PBS + 1 mmol/L Ca^{++}, PBS + 1.5 mmol/L Mg^{++}, or PBS. At five and 30 minutes, the cells were centrifuged for 10 seconds in a microfuge and the supernatants assayed for LDH content. All results are expressed as the percentage of total cellular LDH released and are the mean and standard deviation of quadruplicate samples.

PMN cytoplasts devoid of nuclei and deficient in granules have been used to study activation. We examined the reversible inactivation of cytoplasts by first stimulating them with arachidonic acid followed by albumin or with Con A followed by α-MM. The cytoplast response to these stimuli and inactivators was identical to that seen for whole cells. In addition, reactivation of cytoplasts was no different from that seen for whole cells. Thus, there appears to be no apparent role for ongoing degranulation in the reactivation of the O_2^--generating system by these stimuli.

In many ways, activation of PMN by arachidonic acid is similar to that by other stimuli. In addition, similar to FMLP, O_2^- production by arachidonic acid–treated PMN lasts only a few minutes. However, in contrast to FMLP, cytochalasin B does not enhance the rate or duration of O_2^- production.

We examined the phenomenon of early termination of the O_2^--generating system in detail. We found that the subsequent addition of PMN or arachidonic acid to the reaction mixtures did not result in additional O_2^- production. The addition of both fresh cells and fresh arachidonic acid did result in O_2^- production. The ability of arachidonic acid to activate fresh PMN, even after they had been incubated in the presence of previously activated cells for up to six minutes, indicates that the initial inactivation does not appear to be due to the release of toxic products into the medium. Thus, the early termination by arachidonic acid is probably due both to a depletion of the stimulus (perhaps by incorporation into the PMN) and an irreversible inactivation of the O_2^--generating system.

We also examined the ability of cells treated with arachidonic acid followed by albumin to respond to second stimuli. When albumin was added one minute after activation, O_2^- production could be reinitiated to nearly the maximum attainable rates by the addition of either PMA, Con A, or STZ. Thus, the cells retained their ability to respond to a second stimulus. We also found that, as the time between the addition of arachidonic acid and albumin increased, the ability of the cells to produce O_2^- with the addition of PMA or other stimuli decreased. If the albumin was added after four to five minutes when O_2^- production by arachidonic acid stimulated PMN had ceased, the second stimulus was ineffective at inducing additional O_2^-. This is consistent with the concept that arachidonic acid inactivates the oxidase, the same enzyme it activated. We also compared the effects of activation of PMN by arachidonic acid followed by albumin to PMN activated by Con A followed by α-MM. We activated the cells with the appropriate stimuli and inactivated the oxidase after one or two minutes with albumin or α-MM, respectively. When we added the second stimulus, PMA, at various times after the addition of Con A followed by α-MM, the O_2^--generating system could be reactivated for up to 30 minutes. However, PMN treated with arachidonic acid and albumin in this manner demonstrated a time-dependent inability to be reactivated by PMA. Thus, not only was the continued exposure of PMN to arachidonic acid toxic, but even a brief exposure resulted in a time-dependent inability to respond to a second stimulus.

These phenomena became clarified somewhat when we examined granule enzyme release induced by arachidonic acid. Arachidonic acid–treated PMN released lysozyme in a time-dependent manner. As had been shown for Con A and FMLP, despite reversible inactivation of the O_2^--generating system with arachidonic acid, the addition of albumin did not prevent further release of this enzyme. However, in contrast to Con A–treated PMN, LDH release also occurred. Only for low concentrations of arachidonic acid and for short periods of time was there a greater release of lysozyme than LDH.

Consistent with the aforementioned results are the observations of Badway et al that arachidonic acid stimulation of PMN results in significant morphologic changes indicative of cell damage.[24] However, unlike their observations of morphologic reversibility with albumin, we found continuing evidence of cell damage, even after albumin addition.

The toxicity of arachidonic acid to PMN was further intensified by the addition of EGTA or in the absence of Ca^{++}. This effect of arachidonic acid needs to be kept in mind when analyzing the recent reports on the ability of arachidonic acid to activate the O_2^--generating system in cell-free systems.[34–36] Of note is that this activation occurs in the absence of Ca^{++} and, in one instance, was inhibited by Ca^{++}.[34] Thus, the mechanism of activation of whole cells by arachidonic acid may be very different from the mechanism of activation of cell-free systems. The role(s) of extracellular Ca^{++} in the mechanism of activation of PMN by arachidonic acid cannot be discerned at this time. The toxicity of arachidonic acid to PMN in the absence of Ca^{++} precludes completely evaluating its role.

O'Flaherty et al did not find either LDH or lysozyme release when they incubated PMN with arachidonic acid.[37] However, the highest concentration they used was 10 μmol/L, and they incubated their cells for only five minutes. The combination of the concentration and time used in their study may have precluded the possibility of finding either enzyme released. We were able to demonstrate both a concentration and time dependency on the release of lysozyme and LDH.

Naccache et al did find selective release of lysosomal enzymes from arachidonic acid and cytochalasin B–treated neutrophils at 1 to 100 μmol/L arachidonic acid.[38] They did not find any evidence for LDH release. They used rabbit peritoneal neutrophils, which may have different sensitivity to arachidonic acid. We have previously shown that elicited neutrophils have altered reactivity to phagocytic stimuli and inhibitors when compared with peripheral blood neutrophils.[39]

We conclude that arachidonic acid shares many of the characteristics of other activators of the respiratory burst, including the presence of a lag time, dependence on external calcium, reversibility of activation, and ability to activate cytoplasts. It appears, however, that even a brief exposure of PMN to arachidonic acid results in time- and dose-dependent irreversible damage to PMN. These results and others described previously preclude the possibility of using arachidonic acid to study the latter aspects of the respiratory burst and other late events accompanying stimulus-response coupling.

ACKNOWLEDGMENT

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Red Cell Rheology in Stomatocyte-Echinocyte Transformation: Roles of Cell Geometry and Cell Shape

By Walter H. Reinhart and Shu Chien

The influence of the shape of the red blood cell during stomatocyte-echinocyte transformation on its deformability was studied by microsieving through pores with diameters of 2.6, 4.5, and 6.9 µm. A stomatocytic transformation was produced by chlorpromazine (0.02, 0.1, and 0.5 mmol/L) and an echinocytic transformation by sodium salicylate (7.5, 30, and 120 mmol/L). For spherostomatocytes, an increase in filtration resistance through 2.6 and 4.5 µm pores was observed, whereas for spheroechinocytes, a decrease in filtration resistance through 2.6 µm pores was found. Larger pores (6.9 µm) were not sensitive to those shape changes. The changes in deformability can be explained by the fact that the surface area of (sphero)-stomatocytes decreased, whereas that of (sphero)-echinocytes increased; the cell volume remained essentially constant. Echinocytes produced by 24-hour adenosine triphosphate depletion differed from drug-induced echinocytes: they had an increased cell volume at constant surface area and consequently an increased filtration resistance through 2.6- and 4.5-µm filter pores. Shape changes with spicule formation are therefore not a homogeneous entity, and cell geometric factors (eg, surface area and volume) must be assessed with care. The viscosity of red cell suspensions at a hematocrit level of 45% was higher for drug-induced echinocytes than discocytes or stomatocytes at all shear rates tested. We conclude that the normal discocyte represents an optimum shape for the flow in vivo since a stomatocytic transformation could impair the passage through the microcirculation (decrease in cell filterability) and an echinocytic transformation could impair the flow in larger vessels (increase in blood viscosity).
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THE SHAPE of a normal red blood cell (RBC) is that of a biconcave discocyte It is well recognized that this shape represents an equilibrium state between two opposing extremes of red cell shapes, ie, the stomatocyte and the echinocyte.[1] Red cells can easily undergo either transformation in vitro under the influence of certain agents.[1] These changes are reversible upon the removal of the causative agent or the addition of an antagonist.[1] A stomatocytic transformation is characterized by a tendency of membrane internalization and echinocytic transformation by membrane externalization. It has been suggested that stomatocytic transformation could be caused by a preferential incorporation of the causative agent in the inner hemileaflet of the membrane bilayer and echinocytic transformation by a predominant incorporation in the outer hemileaflet.[2] The red cell volume remains virtually unaltered[3] in both types of transformation.

This stomatocyte-discocyte-echinocyte equilibrium provides a unique in vitro model system to study the influence of RBC shape on the rheologic behavior of blood. The flow properties of blood in large vessels are governed primarily by the hematocrit level, plasma viscosity, RBC aggregation, and RBC deformability, which are the major determinants of whole blood viscosity.[4] The flow of blood in capillaries is primarily dependent on the deformability of the blood cells, which allows RBCs with a diameter of 8 µm to pass through channels with a diameter of 4 to 5 µm. The deformability of RBCs in such narrow channels is determined by the cell geometry, ie, the surface area/volume ratio or sphericity of the RBCs, the viscosity of the cytoplasm, and the biophysical properties of the cell membrane (which can be assessed by micropipette technique).[4] An in vitro test that can be used to assess the ability of red cells to pass through narrow channels is the filterability measurement by microsieving.[5,6]

We used filters with different pore sizes to study the influence of stomatocyte-echinocyte transformation on RBC deformability. The ultimate aim of the study was to obtain results from this in vitro model that allow further investigation of hematologic disorders with an abnormal RBC shape, eg, acanthocytosis, elliptocytosis, spherocytosis, etc.

MATERIALS AND METHODS

Blood was drawn from healthy young volunteers into vacutainer tubes containing 20 United States Pharmacopeia U/mL sodium heparin. After centrifugation at 1,000 g for ten minutes the plasma, buffy coat, and the uppermost RBC layer were removed. The RBCs were washed three times in Ringer's solution containing 0 85 g/dL NaCl, 0.03 g/dL KCl, 0.033 g/dL $CaCl_2$, 0.1 g/dL dextrose, and 0.5 g/dL bovine serum albumin, buffered to pH 7.4 with 0.1 N Tris (final Tris concentration about 0.005 N). Ringer's solution was prefiltered through 0.2-µm Millipore membranes before use. The washed RBCs were resuspended in Ringer's solution at a hematocrit value of 10.0%. The hematocrit value was determined in duplicate by the microhematocrit method. RBC and residual WBC counts were determined with an electronic particle counter (Coulter Counter Model ZB, Coulter Electronics, Hialeah, Fla). For the assessment of hemolysis, the RBC suspensions were centrifuged and the light absorption of the supernatant measured in a spectrophotometer at 540 nm. Reference values were obtained from the same amount of RBCs in distilled water (100% hemolysis) and from cell-free Ringer's solution (0% hemolysis)

Fresh stock solutions of chlorpromazine hydrochloride (CP) and sodium salicylate (SA) were prepared for every experiment The stock CP solution contained 1.5 mmol/L CP in Ringer's solution, with an osmolality of 300 mosm/kg H_2O and pH adjusted to 7.1 with 0.1 N Tris (at higher pH CP was not well soluble); the stock SA solution contained 160 mmol/L SA in distilled water, with an osmolality of 290 mosm/kg H_2O and pH adjusted to 7.4 with 0.1 N Tris The RBC suspension (hematocrit, 10.0%) was divided into

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several aliquots. The aliquots were centrifuged (1,000 g for two to three minutes) to sediment RBCs and generate Ringer's solution supernatant. A calculated amount of Ringer's solution was replaced by an equal volume of either stock solution to obtain CP concentrations of 0.02, 0.1, and 0.5 mmol/L (final pH, 7.3) and SA concentrations of 7.5, 30, and 120 mmol/L. These RBC suspensions were then incubated for 20 minutes at 37 °C and used immediately afterwards for all experiments.

For adenosine triphosphate (ATP) depletion experiments, blood was drawn into sterile, heparinized vacutainer tubes. One half of the sample was used immediately for control measurements on fresh RBCs; the rest was incubated at 37 °C for 24 hours. A 24-hour incubation at 37 °C has been shown to deplete the RBC completely of ATP.[7-9] Fresh and incubated blood samples were centrifuged, the plasma and buffy coat removed, and the RBCs washed three times and resuspended in Tris-buffered Ringer's solution without glucose and albumin (hematocrit, 10%).

The RBC morphology was assessed by scanning electron microscopy (SEM). The RBC suspensions were fixed in 1% glutaraldehyde in cacodylate buffer (pH 7.4, 4 °C), postfixed in 2% OsO_4, dehydrated in ascending ethanol series, air-dried, and coated with gold-palladium. The specimens were viewed at 25 kV in a scanning electron microscope (model JSM-35, Jeol USA, Inc, Peabody, Mass). Some samples were embedded in epon after fixation. Thin sections were prepared and stained with 2% uranyl acetate and lead citrate for transmission electron microscopy (TEM; Model EM 9, Carl Zeiss Inc, Oberkochen, West Germany).

Quantitative assessment of shape transformations was made by the investigator without any knowledge of the type of treatment. Scanning electron micrographs at a magnification of 1,000× were used. In each experiment an average of 150 cells were classified for each type of treatment. The general classification of Bessis[10] was applied with some additional criteria. The stomatocytic transformation was classified as follows: stomatocyte I, a cup-shaped RBC (or convex-concave RBC); stomatocyte II, RBC with a pronounced concavity; stomatocyte III, RBC with a deep concavity and a variable mouthlike distortion and/or pitlike membrane indentations; and spherostomatocyte, a spherical RBC with an irregular area of pits. The echinocytic transformation was classified as follows: echinocyte I, an irregularly contoured discocyte with up to five buckles visible on SEM; echinocyte II, a flat RBC with multiple spicules; echinocyte III, an ovoid or spherical RBC with 30 to 50 spicules; and spheroechinocyte, a sphere with multiple short and thin spicules.

Morphometric analysis was performed on transmission electron micrographs using standard methods.[11] For volume assessments the point-counting method was used, for surface areas the method of intercept counting.

The surface area of RBCs was assessed in a wet preparation by light microscopy (Nomarski Optics; magnification, × 6,925) by using a modified version of the method described by Chabanel et al.[12] The RBC suspension was placed between a glass slide and a coverslip. By adding water from one side, an osmotic gradient was generated in the suspension; as a result, RBCs ranging from the initial shape to various degrees of swelling and hemolysis could be observed along the osmotic gradient created. There existed a sharp interface between maximally swollen cells and cells already hemolyzed; the diameter of these spherical cells along that line of interface was measured and the surface area calculated. Using the mean corpuscular volume measured prior to swelling, the sphericity index[13] was calculated (sphericity index $= 4.84 \times V^{2/3}/A$, where V and A are the volume and surface area of the cell, respectively). A perfect sphere has a sphericity index of 1.0.

The RBC osmotic resistance was measured with a routine procedure.[14] The packing capacity of stomatocytes and echinocytes was studied by ultracentrifugation of RBC suspensions at 100,000 g for 15 minutes. This supernatant was replaced by 1% glutaraldehyde, followed by another centrifugation of 15 minutes at 100,000 g. After overnight fixation, the RBCs were processed for TEM.

Red cell filtration was performed as described elsewhere.[6] In brief, the RBC suspensions were placed in a glass syringe and pumped at a constant flow rate of 0.82 mL/min (Model 975, Harvard Apparatus Co, Inc, Millis, Mass) and a temperature of 37 °C through polycarbonate filters (Nucleopore Corp, Pleasanton, Calif) with different pore sizes. The filters had a diameter of 13 mm, a thickness of 13 μm, and nominal pore sizes of 3 μm (lot no. 62C1A70, mean ± SD pore diameter determined by SEM to be 2.6 ± 0.2 μm), 5 μm (lot no. 54C1D7, pore diameter 4.5 ± 0.6 μm) and 8 μm (lot no. 51C9A29, pore diameter 6.9 ± 0.8 μm). The filtration pressure during constant flow was measured on the upstream side close to the filter with a pressure transducer (model MP 45-14, Validyne Engineering Corp, Northridge, Calif) connected to an amplifier (Validyne, model MC 1-3) and a recorder (model 385, Linear Instruments Corp, Costa Mesa, Calif). Prior to the filtration of RBC suspensions, Ringer's solution containing the same concentrations of CP or SA was filtered to obtain the filtration pressure for suspending medium (P_o); the presence of CP or SA did not affect the P_o value. The initial pressure rise (P_i), which reflects the deformability of the RBCs in suspension,[15] was determined for the RBC suspensions. The relative filtration resistance of an individual red cell, β, was calculated as follows: $\beta = 1 + [(P_i/P_o) - 1]V/h$, where V is the fraction of the pore volume[16] occupied by the red cell and h is the fractional volume of RBCs in suspension.[6,16] This dimensionless parameter β, developed by theoretical modeling,[16] is the ratio of resistance in a pore bearing a RBC to that filled with buffer alone.

The viscosity of suspensions of RBCs in Ringer's solution containing the drug (CP or SA) was measured with a Couette viscometer,[17] ie, a rotational cylinder viscometer, at various shear rates from 0.1 to 208/s.

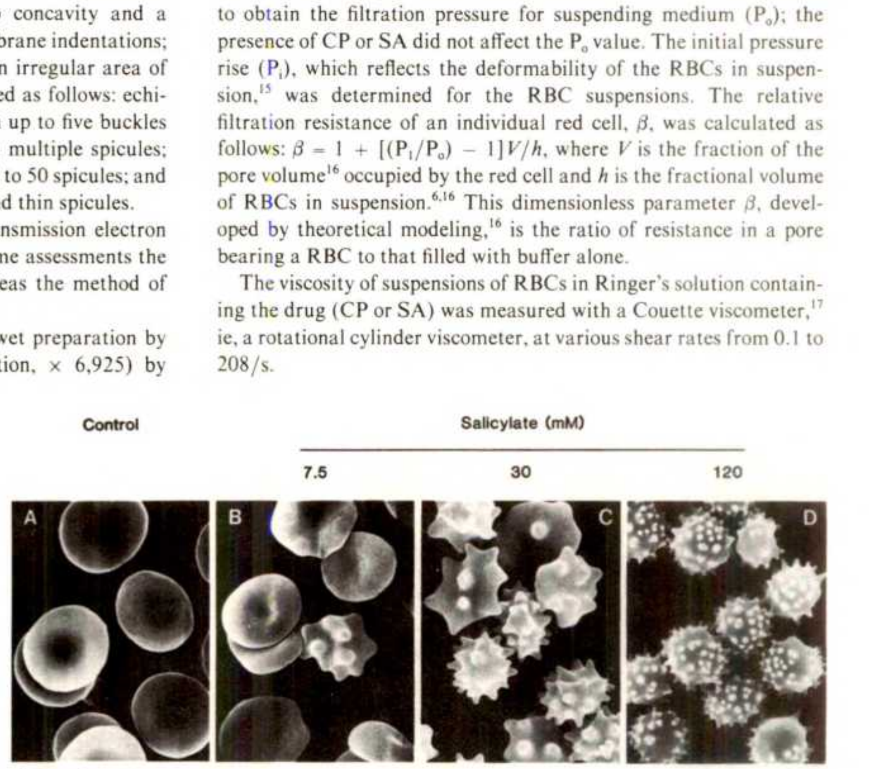

Fig 1. Scanning electron micrographs of RBCs undergoing echinocytic transformation induced by SA. (A) Normal discocytes. (B) SA, 7.5 mmol/L, with discocytes and echinocytes I. (C) SA, 30 mmol/L, with primarily echinocytes II. (D) SA, 120 mmol/L, with spheroechinocytes.

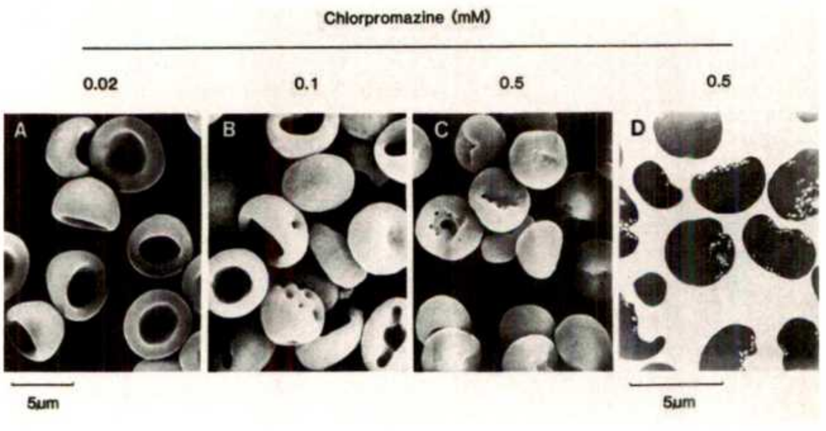

Fig 2. Scanning electron micrographs of red cells showing a dose-dependent stomatocytic transformation from stomatocytes I and II with 0.02 mmol/L chlorpromazine (A), to stomatocytes II and III with 0.1 mmol/L CP (B), and to spherostomatocytes with 0.5 mmol/L CP (C). (D) Transmission electron micrograph showing spherostomatocytes (0.5 mmol/L chlorpromazine) with multiple membrane-coated vacuoles within the cytoplasm.

For statistical analysis a one-way analysis of variance was used. Data on ATP-depleted RBCs were analyzed by Student's *t* test.

RESULTS

The morphologic changes of red cells after incubation with different doses of SA are shown in Fig 1. A concentration of 7.5 mmol/L SA had a discernible effect on red cell morphology (Fig 1B, discocytes and echinocytes I), 30 mmol/L SA induced a clear echinocytic transformation with predominantly echinocytes II (Fig 1C), and 120 mmol/L SA transformed the vast majority of red cells into spheroechinocytes (Fig 1D). The dose-dependent stomatocytic transformation obtained with chlorpromazine is shown in Fig 2. CP at 0.02 mmol/L generated mainly cup-shaped RBCs (Fig 2A), 0.1 mmol/L CP predominantly stomatocytes II and III (Fig 2B), and 0.5 mmol/L CP spherostomatocytes (Fig 2C). Spherostomatocytes had multiple intracellular vacuoles as seen by TEM (Fig 2D). When the extracellular marker ruthenium red[18] was added to the cell suspensions prior to embedding, the vast majority of the vacuoles (more than 95%) were ruthenium red–negative, indicating that these vacuoles were not in communication with the cell surface and the extracellular fluid. By morphometric analysis it was calculated that 3% of the total cellular volume was occupied by vacuoles. The degree of transformation was assessed in three to four different experiments by classifying the shape of 150 to 200 RBCs on SEM pictures. As shown in Table 1, the concentrations of the two antagonistic drugs chosen in this study cover the whole range of transformation from spherostomatocytes to spheroechinocytes. Spherostomatocytes and spheroechinocytes were not completely reversible, but lesser degrees of transformation fully recovered upon removal of the drug or by adding the antagonist.

Examples of pressure-time curves obtained for discocytes, stomatocytes, and echinocytes are shown in Fig 3. The filtration pressure curves obtained with 4.5-μm pores were not significantly affected by SA, even with the formation of spheroechinocytes in 120 mmol/L SA (Fig 3A). CP at concentrations of 0.02 and 0.1 mmol/L also did not have a significant effect on the filtration pressure curves with 4.5-μm pores, but at 0.5 mmol/L, CP elevated the pressure-time curve approximately fivefold. With 2.6-μm pores (Fig 3B), the slope of the pressure-time curve was more flat for echinocytes (30 and 120 mmol/L SA) and steep for stomatocytes (0.1 and 0.5 mmol/L CP). It is possible that the RBC passage did occur primarily through pores with a diameter larger than 2.6 μm (about 12% of the pores are >2.8 μm, ref 6). No differences were found for 6.9-μm pore filters (not shown). The initial pressure (P_i) was ascertained on these pressure tracings (Fig 3). For 2.6-μm pores and 0.5 mmol/L CP, the transition of the pressure-time curve (P_i) was not always clearly definable (Fig 3B); P_i was therefore defined for 2.6-μm pores as the pressure reading obtained 2 seconds after the onset of the pressure rise,[19] a time when P_i was normally found for other CP and SA concentrations.

The ratio β of the resistance in a pore bearing an RBC in transit to that in a pore with Ringer's solution alone was calculated. The results are shown in Figure 4. For the narrow

Table 1. Percentage of Red Cell Shapes

	Chlorpromazine (mmol/L)				SA (mmol/L)		
	0.5	0.1	0.02	0	7.5	30	120
Spherostomatocytes	92 ± 14	1 ± 1					
Stomatocytes III	8 ± 14	21 ± 6					
Stomatocytes II		44 ± 8	8 ± 8				
Stomatocytes I		28 ± 13	52 ± 28	18 ± 10	5 ± 4		
Discocytes		6 ± 4	40 ± 22	78 ± 11	63 ± 10	2 ± 2	
Echinocytes I				4 ± 5	32 ± 14	17 ± 11	
Echinocytes II						53 ± 7	2 ± 4
Echinocytes III						28 ± 7	9 ± 16
Spheroechinocytes							88 ± 20

Values are the mean ± SD.

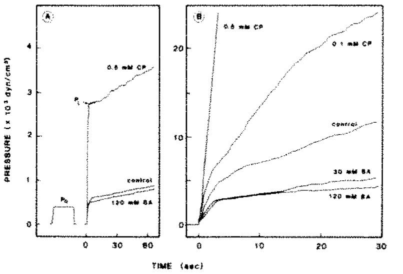

Fig 3. Pressure-time curves obtained for the filtration of suspensions with a fixed number of RBC ($0.98 \times 10^6/\mu L$) at a flow rate of 0.82 mL/min (resulting in a cell flux of 8×10^8 cells/min) through filters with a pore diameter of 4.5 μm (A) and 2.6 μm (B). Controls, stomatocytes (0.1 and 0.5 mmol/L CP), and echinocytes (30 and 120 mmol/L SA) are shown. P_0 is the pressure reading for Ringer's solution alone, P_i is the initial pressure for the RBC suspension.

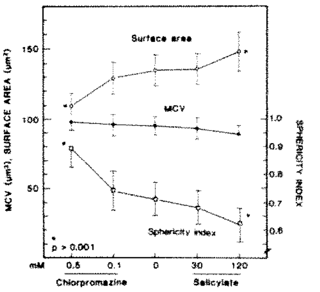

Fig 5. Geometric characteristics of stomatocyte-echinocyte transformation. The mean corpuscular volume (MCV, mean ± SD of six experiments), the surface area measured after swelling of the cells to obtain a spherical shape, and the calculated sphericity index for this experiment are shown for stomatocytes (0.1 and 0.5 mmol/L CP), discocytes, and echinocytes (30 and 120 mmol/L SA).

filter pores with a diameter of 2.6 μm, the increasing degree of (sphero) stomatocytosis induced by CP tended to increase the filtration resistance (β) of these red cells, whereas the increasing degree of (sphero-)echinocytosis induced by SA tended to decrease β; the changes being statistically significant at the highest concentrations of SA and CP used. Spherostomatocytes (0.5 mmol/L CP) also caused a significant increase in β for 4.5-μm pores, but filtration through 6.9-μm pores was insensitive to these shape changes.

Hemolysis in the suspensions before and after filtration was less than 1% for all shape transformations except spherostomatocytes (0.5 mmol/L CP) where hemolysis was 0.5% before filtration and 80% in the filtrate.

The geometric characteristics of stomatocytes and echinocytes are shown in Fig 5. The MCV showed a trend to be slightly higher in 0.5 mmol/L CP and slightly lower in 120 mmol/L SA as compared with discocytes; the difference was not statistically significant. The surface area of normal RBCs measured with our swelling method was 135 ± 12 μm^2; this agrees with the results of Linderkamp et al[20] who obtained 134 ± 14 μm^2 with the micropipette method. The RBC surface area was lower for 0.5 mmol/L CP and higher for 120 mmol/L SA. The intracellular vacuoles seen in spherostomatocytes (Fig 2D) may account for the loss in surface area. Spheroechinocytes, incubated at 135 mosm/kg H_2O for maximum swelling, were examined by TEM. It was found that all spicules were smoothened out by that procedure (not shown). The sphericity index was significantly higher for RBCs in 0.5 mmol/L CP and lower for RBCs in 120 mmol/L SA (Fig 5). This was confirmed by morphometric analysis of TEM pictures of spherostomatocytes and spheroechinocytes (not shown).

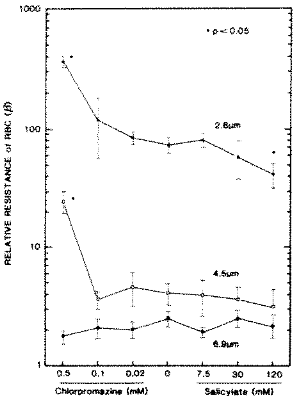

Fig 4. Semilogarithmic plot of the relative filtration resistance of an RBC (β) *v* different degrees of stomatocytic transformation (0.02, 0.1, and 0.5 mmol/L chlorpromazine) and echinocytic transformation (7.5, 30, and 120 mmol/L salicylate) for pore diameters of 2.6, 4.5, and 6.9 μm. The results represent the mean ± SD of four experiments.

The difference in sphericity between spherostomatocytes and spheroechinocytes is illustrated in Fig 6. Spheroechinocytes showed a capacity to deform in centrifugal packing, with the disappearance of spicules (Fig 6B), and the transmission electron micrographs of centrifugally packed spheroechinocytes are similar to those of the packed discocytes.[21] Packed spherostomatocytes were composed of undeformable spheres, and a significant degree of centrifugal packing was attained by the intercalation of less severely transformed cells that still had sufficient deformability to fit between the spheres (Fig 6A).

The relationship between cell surface area and volume was further tested by determining the osmotic resistance (Fig 7). The osmotic resistance was increased for echinocytes (30 and 120 mmol/L SA) and decreased for stomatocytes (0.1 mmol/L CP). For spherostomatocytes (0.5 mmol/L CP) complete hemolysis occurred above 0.6% saline.

The RBC morphology observed after metabolic depletion by incubating whole blood for 24 hours at 37 °C is shown in Fig 8A. The cell shape ranged from discocyte to echinocyte III. When these cells were washed and incubated in Ringer's solution containing 0.5 g/dL albumin, they regained a discoid shape (Fig 8B), which has also been observed by Ferrell and Huestis.[9] Sometimes the shape reversal even showed an overshoot, resulting in cup-shaped RBCs. Depleted RBCs washed and resuspended in buffered saline remained echinocytes (Fig 8C); the degree of echinocytic transformation became even more pronounced when the depleted RBCs were transferred from plasma to saline (Fig 8A). Fresh cells incubated in buffered saline for 20 minutes at 37 °C showed no echinocytic transformation (Fig 8D).

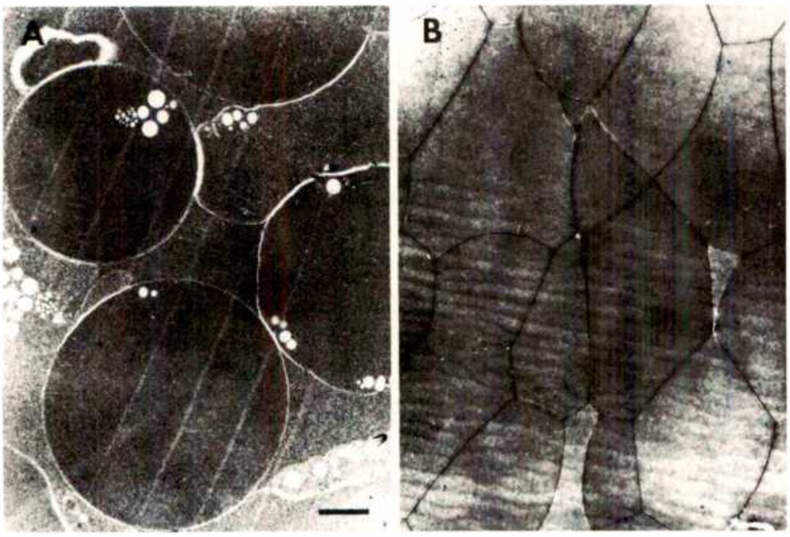

Fig 6. Transmission electron micrographs of (A) spherostomatocytes (0.5 mmol/L CP) and (B) spheroechinocytes (120 mmol/L SA) fixed with 1% glutaraldehyde during centrifugation at 100,000 g. Bar represents 1 μm.

The results of filtration studies on 24-hour–depleted RBCs washed and resuspended in Tris-buffered saline are shown in Fig 9. The relative filtration resistance (β) in 2.6-μm pores for the echinocytes induced by 24-hour depletion was more than twice as high as that for the discocytes from the same donor studied immediately after blood withdrawal. β was slightly but significantly increased for 4.5-μm pores also, whereas 6.9-μm pores were insensitive.

The geometry of the depleted RBCs is shown in Fig 10. The 24-hour depletion did not affect the surface area, slightly increased the cell volume, and therefore increased significantly the sphericity index ($P < .05$). These geometric results, as the filtration data, are in contrast to those obtained with drug-induced echinocytosis.

The morphology of RBCs was unaffected by the filtration procedure for stomatocytes as well as echinocytes (Fig 11). This indicates that despite the differences in filtration resistance there was no noticeable selective retention of cells with a higher filtration resistance by the filter and that the shape transformation is not altered after the mechanical shearing during filtration.

Neither drug affected the viscosity of the suspending medium alone (the same value of 0.75 CP for control, 0.5 mmol/L CP, and 120 mmol/L SA). Viscosity measurements of RBC suspensions in Ringer's solution (Fig 12) showed that spheroechinocytes (120 mmol/L SA) had a significantly higher viscosity than discocytes (control) and spherostomatocytes (0.5 mmol/L CP). The difference was more pronounced at low shear rates.

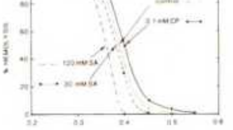

Fig 7. Osmotic resistance of discocytes (control), echinocytes (30 and 120 mmol/L SA), and stomatocytes (0.1 mmol/L CP). For spherostomatocytes (0.5 mmol/L CP), complete hemolysis occurred above the upper limit of 0.6% saline shown in this graph.

DISCUSSION

Stomatocytic and echinocytic transformations of red blood cells are dose dependent. The degree of transformation for a given drug concentration found in the present study agrees with previous reports.[3,22] The concentrations used for shape transformations are higher than the therapeutic plasma concentrations, which are about 0.001 mmol/L for chlorpromazine[23] and 2 mmol/L for salicylate.[24]

The stomatocyte-echinocyte transformation of RBCs affected their filterability through narrow pores. The sensitivity of the filtration test increased with decreasing filter pore size, which is in agreement with a previous study.[6] For a pore size of 2.6 μm, spherostomatocytic transformation (0.5 mmol/L CP) increased the filtration resistance, ie, decreased RBC deformability, whereas spheroechinocytic transformation (120 mmol/L SA) increased RBC deformability in this system. These changes in deformability can be explained by differences in surface area and cell volume. With spherostomatocytic transformation (0.5 mmol/L CP) membrane vacuoles were formed, which became detached from the cellular membrane towards the cell interior. The formation of intracellular membrane vacuoles reduced the membrane surface area and increased the cell volume by the volume of the vacuoles filled with suspending medium. Morphometric evaluation revealed that 3% of the cell interior of RBCs treated with 0.5 mmol/L CP was occupied by vacuoles, which alone can account for the 3% increase in MCV (Fig 5). The loss of surface area and the gain in volume because of such vacuole formation combined to cause the increase in sphericity, resulting in an increase in the sphericity index to 0.93.

Spheroechinocytes produced by 120 mmol/L SA also had an overall spherical appearance. But they were not spherocytic in a strict sense since the abundant number of little spicules lead to an increase of surface area by about 10%. This excess surface area was available for different types of

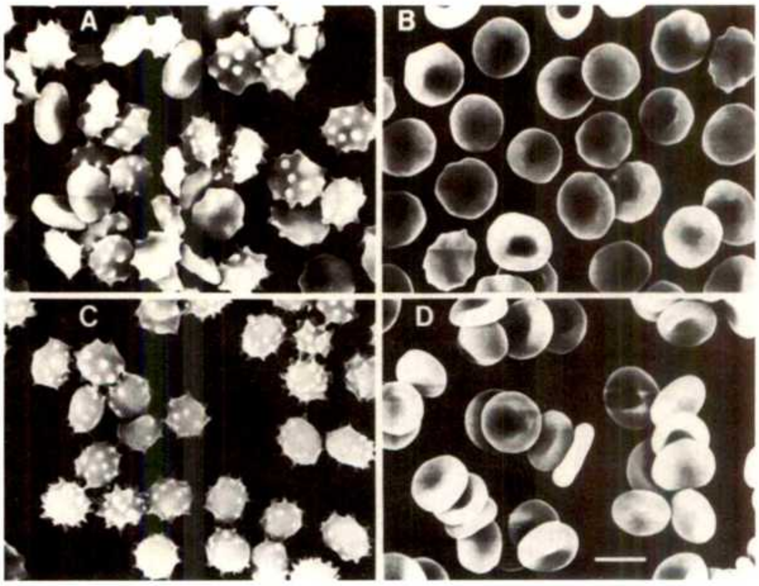

Fig 8. Scanning electron micrographs of 24-hour–depleted RBCs. (A) RBCs from whole blood incubated for 24 hours at 37 °C. (B) The same RBCs as in (A) suspended in Ringer's solution containing 0.5 g/dL bovine albumin. (C) RBCs of (A) suspended in Tris-buffered saline without albumin. (D) Fresh RBCs suspended in Tris-buffered saline for 20 minutes at 37 °C.

cellular deformation, eg, during osmotic swelling of the cells (Fig 7), RBC packing (Fig 6), and filtration. It has been reported that the membrane expansion produced by amphiphilic drugs that are incorporated into the membrane is several times greater than the pure volume of occupation within the membrane.[25] This has been explained on the basis of disordering and expansion of lipid regions, conformational changes, rearrangement of membrane proteins, and/or a change in membrane calcium content.[25] It is possible that these changes, in addition to the incorporation of SA into the membrane, contributed to the 10% membrane expansion found in our study. The volume of spheroechinocytes decreased, although it did not reach statistical significance. This is probably the result of the Gardos effect; salicylate increases the permeability for calcium, which activates calcium-sensitive potassium channels, leading to a loss of potassium and water from the RBC.[26]

Stomatocyte-echinocyte transformation has not been studied systematically with the RBC filtration test. It has been reported briefly that stomatocytes produced by chlorpromazine had a higher filtration pressure than normal discocytes.[27] Other methods that test the deformability of the whole cell have been used. Leblond[28] measured the pressure required to aspirate whole echinocytes into micropipettes with a diameter of 2.8 to 3.0 μm. This approach can be compared with the 2.6-μm pore filters of our study. It was found that echinocytes produced by lysolecithin and pH 8.5 required less pressure than discocytes. This finding is consistent with the lower filtration resistance following SA treatment found in our experiment. Leblond[28] also found that echinocytes produced by oleate required the same pressure as discocytes; echinocytes produced by ATP depletion required pressures ten times higher.

Another approach to the measurement of RBC deformability is to use a system without geometric constraints on individual RBCs as in narrow pores, eg, ektacytometry,[29] high speed centrifugation,[30] and the counterrotating rheoscope.[30] Using these methods it was found that stomatocytes produced by chlorpromazine are less deformable than discocytes[29,30] and that spherostomatocytes are undeformable,[29] which is in agreement with our results with filter pore sizes of 2.6 and 4.5 μm. Echinocytes III had a similar deformability in ektacytometry as discocytes, but spheroechinocytes were less deformable.[29] With high-speed centrifugation and the rheoscope, echinocytes were less deformable than discocytes.[30] The discrepancy between these findings and our results might be due to differences in methodology in the

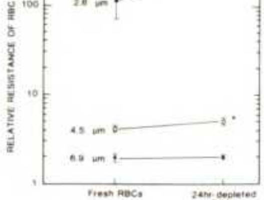

Fig 9. Relative filtration resistance of individual red blood cells, β, for fresh RBCs and 24-hour–depleted RBCs for filters with 2.6-, 4.5-, and 6.9-μm pore diameter. Values represent means ± SD, n = 4.

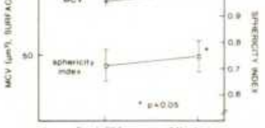

Fig 10. Surface area, MCV, and calculated sphericity index for fresh and depleted RBCs (24 hours at 37 °C). Values represent means ± SD.

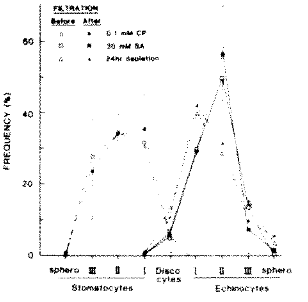

Fig 11. **Red blood cell morphology before and after filtration through Nucleopore filters with a nominal pore diameter of 3 μm for suspensions of red blood cells treated with 0.1 mmol/L chlorpromazine and 30 mmol/L salicylate (three experiments each) and red blood cells depleted for 24 hours at 37 °C (one experiment).**

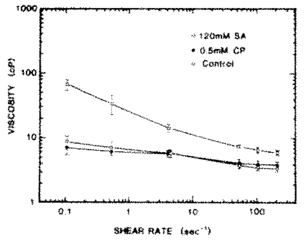

Fig 12. **Viscosity plotted against shear rate for suspensions of spheroechinocytes (120 mmol/L SA), spherostomatocytes (0.5 mmol/L CP), and discocytes (control). The hematocrit level was 45%. Values represent means ± SD, n = 4.**

three studies, eg, the use of different agents (lysolecithin in ref 29 and 2,4-dinitrophenol in ref 30) to produce echinocytes and the use of the highly viscous dextran solutions in ektacytometry[29] and the rheoscope.[30] Deformability tests with geometric constraints such as filtration through narrow pores might be more influenced by the cell geometry,[31] eg, the surface/volume ratio,[19] than those without geometric constraints (eg, deformation in a laminar flow field studied by ektacytometry and rheoscopy). The shear stresses applied in ektacytometry[29] and filtration[6] are similar (about 100 dyn/cm^2) and do not explain the differences. Spicules have been found to disappear at shear stresses above 10 to 15 dyne/cm^2.[30]

Although the changes in RBC deformability are explained by the changes in the relationship between surface area and volume, the other determinants of red blood cell deformability, ie, the cytoplasmic viscosity and the viscoelastic properties of the membrane, need be considered also. The corpuscular hemoglobin concentration, which determines primarily the cytoplasmic viscosity, did not change significantly in our study; in the case of spheroechinocytes, although there was a slight increase in the mean corpuscular hemoglobin concentration and an expected increase in cytoplasmic viscosity, the filtration resistance was reduced rather than increased. We are not aware of any study on the influence of chlorpromazine and salicylate on membrane deformability. It has been shown, however, that the filtration of RBCs through small pores is primarily affected by the cell geometry.[19] It is therefore likely that the increased sphericity of spherostomatocytes causes the increased filtration resistance and that direct effects of CP on the cell membrane are less important. Leblond[28] found that echinocytes produced by oleate or pH 8.5 required twice the negative pressure for a penetration of 1 μm into a micropipette with a diameter of 1.5 μm, indicating a reduced membrane deformability of echinocytes.

From this filtration study, which has a reasonable in vitro analogy of the flow through capillaries in vivo, it appears that echinocytes, at least those produced with SA, would pass more easily through the capillaries than normal discocytes. The question arises as to why the normal RBC is not echinocytic. Echinocytes have a rheologic disadvantage over discocytes, ie, suspensions of echinocytes have a higher viscosity (Fig 12). This is in agreement with an earlier communication by Meiselman[3] who attributed the increased viscosity to an enhanced cell-cell interaction or tangling of spicules. The increase in viscosity was more pronounced at low shear rates than at high shear rates. At high shear rates the surface of the echinocyte is gradually smoothened out by the high shear forces, as documented by direct observation in a rheoscope.[30] The high viscosity of echinocyte suspension would affect the bulk flow in larger blood vessels. It has recently been shown that echinocytes have also an impaired capacity of oxygen release.[32] The decrease in the rate of oxygen release was pronounced at low flow velocities, probably because of a stagnant unstirred layer around the echinocyte. At high flow velocities when the surface was shown to smooth out, the oxygen-releasing capacity returned toward normal.

Probably the best known cause of echinocytic transformation is ATP depletion. When red blood cells are metabolically depleted in vitro for 24 hours or longer, they become echinocytes.[7] The whole-cell deformability of those ATP-depleted echinocytes has been studied by several investigators, and there is general agreement that they are less deformable.[28,33] This is opposite to our results on echinocytes induced by salicylates. Therefore we performed experiments with 24-hour–depleted RBCs, and the results confirmed the earlier observations of a reduced deformability of these cells (Fig 9). The reason for the discrepancy between the two types of echinocytes may be explained by the fact that the sphericity index increased in ATP-depleted cells, whereas it decreased in salicylate-induced echinocytes. RBCs with the same shape may therefore have different surface area–to-volume relationships and consequently different deformabilities.

The increase in sphericity following ATP depletion was mainly the result of a cell swelling while the surface area remained unchanged (Fig 10); this could be explained by a breakdown of the sodium pump after ATP depletion. Meiselman found the discocyte-echinocyte transformation by ATP depletion to be isovolumic.[30] The difference may be due to the fact that he incubated washed RBCs in isotonic buffer solution, whereas we incubated unwashed RBCs in autologous plasma. Earlier communications support our result of an increased cell volume after metabolic depletion.[34,35] After incubations of >30 hours, a Gardos effect is observed, ie, losses of potassium and water from the RBCs.[8] The 24-hour depletion in this study did not affect the membrane surface area. It is known, however, that a longer depletion period during blood storage leads to a loss of membrane because of exovesiculation of the membrane.[36] This process would additionally increase sphericity and further decrease the filterability of these cells.

We conclude that echinocytic transformation can either be accompanied by an increase in sphericity, which leads to a decrease in deformability, or by a decrease in sphericity, which causes an increase in deformability. The sphericity and not the shape of a red cell appears to be the more important determinant of RBC passage through narrow channels. Stomatocytic transformation and ATP depletion cause an increase in sphericity and a decrease in red cell deformability. Salicylate-induced echinocytic transformation causes a reduction in sphericity and an increase in deformability. Red cell deformability determines not only the rheology of the circulating blood but also the life span of the individual red blood cell.[37] Our results suggest that the discocyte or shapes close to it represent an optimum shape for the in vivo situation. Stomatocytes with their decreased deformability in narrow channels are probably removed more rapidly by the spleen. The same holds true for echinocytes with increased sphericity as seen after metabolic depletion. Echinocytes with unchanged or even decreased sphericity are probably not removed by the spleen more rapidly, but they impair blood flow because of cell-cell interactions and hence increase blood viscosity.

These results may serve as a basis to study other hematologic disorders with an altered red blood cell shape, eg, hereditary elliptocytosis, spherocytosis, acanthocytosis, as well as acquired disorders with spiculed RBCs.[34]

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Expression of Specific Granule Markers on the Cell Surface of Neutrophil Cytoplasts

By Pamela R. Petrequin, Robert F. Todd III, James E. Smolen, and Laurence A. Boxer

The widespread assumption that cytoplasts generated from human polymorphonuclear leukocytes (PMNs) are vesicles consisting solely of cytoplasm surrounded by plasma membrane and devoid of granule activity remains to be tested. PMN cytoplasts were prepared by centrifugation of intact cells on a Ficoll step gradient in the presence of cytochalasin B. Two granule membrane markers, Mol, a fluorometrically detectable antigen, and cytochrome b, both of which have been shown to translocate to the plasma membrane during granule release, were compared for their activity in cytoplasts and intact PMNs. We found that the amount of Mol detected on the plasma membrane of intact PMNs, as compared with other membrane markers (such as antigens LFA-1 and β_2m), increased 1.6-fold upon exposure of PMNs to Ficoll plus cytochalasin B prior to centrifugation. Another twofold increase in Mol expression occurred upon cytoplast preparation. Release of the granule enzymes, vitamin B_{12}-binding protein, and lysozyme were also followed and correlated well (r = .78 and .92) with the amount of Mol antigen present on the cell surface. Cytochrome b was also found to be higher (1.4-fold) on plasma membranes isolated from cytoplasts than on plasma membranes isolated from intact control cells. These results indicate that some fusion of granule membranes and plasma membranes occurred during treatment of PMNs with Ficoll plus cytochalasin b and during cytoplast preparation.

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IT HAS NOT YET been demonstrated that cytoplasts generated from human polymorphonuclear leukocytes (PMNs) are vesicles consisting solely of cytoplasm surrounded by unmodified plasma membrane. The preparation of cytoplasts described by Roos et al[1] involves the centrifugation of cells on a Ficoll step gradient containing cytochalasin b at 34°. They showed that the isolated material consists of vesicles in which 41% of lactate dehydrogenase (LDH) and 33% of alkaline phosphatase activities were recovered.[1] Few granule contents were found in this fraction. Functionally, these vesicles were stimulatable by PMA to consume oxygen, and to produce superoxide and were able to phagocytize opsonized *Staphylococcus aureus*.[1] Others have found these particles to aggregate in response to *N*-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe).[2]

Although Roos et al demonstrated that cytoplasts are deficient in soluble granule markers, their content of granule membrane markers has not been thoroughly examined. Two markers that are believed to be on granule membranes and have been shown to translocate to the plasma membrane after stimulation are Mol[3] and cytochrome b[4] Mol is a membrane antigen that is detectable on human monocytes, PMNs, and null cells.[5] It is believed to be an antigenic site on the C3bi receptor.[6] In immunoprecipitation studies[3] using anti-Mol monoclonal antibody, this antigen was localized to the fraction that has been shown to contain specific granules.[7] Translocation of Mol was demonstrated following stimulation with the calcium ionophore A23187, which caused a five- to tenfold increase in Mol expression on the plasma membrane accompanied by a significant amount of degranulation.[3]

Cytochrome b is another granule marker that has been shown to translocate to the plasma membrane of human PMNs following stimulation of the respiratory burst by soluble agents or opsonized particles.[4,7] There are some indications that cytochrome b is a component of the NADPH oxidase system because it is reduced after stimulation under anaerobic conditions.[8] However, attempts to directly correlate the translocation of cytochrome b activity with increases in oxidase activity upon stimulation have not been conclusive.[4,9,10]

The purpose of these experiments was to examine the claim that cytoplasts are truly free of granule activity. The approach used was to compare membranes from cytoplasts with those from intact control cells with respect to the aforementioned granule membrane markers. Our data indicate that degranulation occurred during cytoplast preparation and that plasma membranes of cytoplasts had significantly higher contents of Mol and cytochrome b than those of intact cells.

MATERIALS AND METHODS

Chemicals Ionophore A23187 was purchased from Calbiochem-Behring Corp, La Jolla, Calif. Ficoll 70 and Percoll were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. (^{57}Co)-vitamin B_{12} and (γ-^{32}P)-ATP were purchased from Amersham Corp, Arlington Heights, Ill. All other reagents were purchased from Sigma Chemical Co, St Louis, unless otherwise stated A23187 and cytochalasin B were dissolved in dimethyl sulfoxide and then added to aqueous solutions with vigorous mixing.

Monoclonal antibodies. Mouse ascites containing monoclonal antibodies specific for LFA-1[11] (IgG1 subclass, a gift from Dr Hergen Spits, Amsterdam, Holland), Mol[5] (IgG2a subclass), β_2m[12] (IgG2b subclass, a gift of Dr Lee Nader, Boston), and Ia[13] (IgG2a subclass) were used in indirect immunofluorescence analysis.

Preparation of cells Suspensions of PMNs were prepared from heparinized (10 U/mL) venous blood from healthy adult donors. Standard techniques of Hypaque-Ficoll gradients[14] followed by dextran sedimentation and hypotonic lysis of erythrocytes provided a PMN suspension that was 98% pure. Neutrophils were washed in phosphate buffered saline (PBS) (138 mmol/L NaCl, 2.7 mmol/L

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KCl, 8.1 mmol/L Na_2HPO_4, and 1.5 mmol/L KH_2PO_4, pH 7.4) before use.

Cytoplast preparation. Cytoplasts were prepared according to Roos et al.[1] In brief, washed PMNs were resuspended in incubation medium (PBS, pH 7.4, with 0.6 mmol/L $CaCl_2$, 1.0 mmol/L $MgCl_2$, 5.5 mmol/L glucose, and 0.5% bovine serum albumin [BSA]) and incubated for five minutes at 37 °C. The suspension was centrifuged at 700 g at room temperature for six minutes. The cell pellets were resuspended in 12.5% Ficoll with 20 µmol/L cytochalasin b, incubated for five minutes at 37 °C, and then layered on a Ficoll step gradient (16%/25%: 5.5 mL of each containing 20 µmol/L cytochalasin b). This gradient was centrifuged at 81,000 g at 34 °C for 30 minutes in a Beckman SW 28.1 rotor in a Beckman L65-B ultracentrifuge (Beckman Instruments, Inc, Fullerton, Calif). The materials at the interfaces were collected and then washed twice in PBS, pH 7.4, with 10 mmol/L $CaCl_2$ and 10 mmol/L $MgCl_2$.

Plasma membrane preparation. Approximately 2.5 × 10^8 washed PMN were resuspended in 12 mL of relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 3.5 mmol/L $MgCl_2$, and 10 mmol/L piperazine-*N*, *N*¹-bis(2-ethanesalfonic acid), pH 7.3) and pressurized under N_2 for 20 minutes with stirring in a nitrogen bomb[15] The cavitate was collected dropwise into ethylene glycol tetraacetic acid for a final concentration of 1 mmol/L For the isolation of plasma membranes, the procedure of Borregaard et al[7] was followed with minor changes. Fourteen milliliters of a 1.044-g/mL Percoll solution were added to a 50-mL polycarbonate tube (Beckman, Irvine, Calif), and 14 mL of the 1.11-g/mL Percoll were layered using a spinal needle The cavitated sample, 8.0 to 10.0 mL, was layered on top and centrifuged at 4° for 17 minutes at 20,000 rpm in a Beckman JA-20 rotor using a Beckman J2-21M centrifuge The bands of material at the two interfaces, γ and β, and the pellet at the bottom, α, were collected. Percoll was removed by centrifugation at 100,000 g for 105 minutes at 4 °C in a Beckman SW 41 rotor. Material lying on top of the Percoll pellet was collected with a Pasteur pipette.

Enzyme marker analysis. Alkaline phosphatase was assayed according to DeChatelet and Cooper,[16] with 10 mmol/L *p*-nitrophenol phosphate (Sigma) in a 50 mmol/L glycine buffer, pH 10, with 10 mmol/L $MgCl_2$. The enzyme activity was calculated using the extinction coefficient for *p*-nitrophenol (18.6/L · $mmol^{-1}$ · cm^{-1}).[17]

Vitamin B_{12}–binding protein was measured by a modification of the method of Gottlieb et al.[18] Sixteen picograms of (^{57}Co)–vitamin B_{12} in 0.6 mL of saline with 200 pg of carrier vitamin B_{12} was added to the protein sample in 1.0 mL of saline for a final specific activity of 90 cpm/pg The reaction was run for 30 minutes at room temperature after which 1.0 mL of 2.5% charcoal, 0.5% vitamin B_{12}–deficient BSA (Sigma) in 0.9% NaCl was added to stop the reaction. Samples were centrifuged for ten minutes at 800 g at room temperature, and 1.0 mL aliquots of the supernatants were collected and counted in a Tractor Analytic Model 1190 (TM Analytic, Elk Grove, Ill) gamma counter to determine the amount of bound (^{57}Co)-B_{12}

LDH was measured as described by Wacker et al[19] Lysozyme was measured turbidometrically[20] using egg white lysozyme (Sigma) as a standard. β-Glucuronidase was assayed using phenolphthalein β-D-glucuronide as a substrate.[21] Activity was calculated using an extinction coefficient of 33 L · $mmol^{-1}$ · cm^{-1} at 540 nm.[22] Mg–adenosine triphosphatase (Mg-ATPase) was assayed as previously described.[23] Myeloperoxidase was assayed according to the procedure of Bretz and Baggiolini.[24] Protein was measured according to the Bradford procedure.[25]

Cytochrome b determination. Cytochrome b was assayed by performing difference spectra from 600 to 400 nm on samples sonicated in PBS[26] Scans of samples reduced in sodium dithionite minus background scans of oxidized sample produced characteristic cytochrome b peaks at 559 nm and 428 nm Although the 559-nm peak was more characteristic of cytochrome b activity, the 428-nm peak was larger and a more sensitive measure. Cytochrome b was quantitated using an extinction coefficient of 106 L · $mmol^{-1}$ · cm^{-1} for the 428-nm peak.[9]

Immunofluorescent analysis of membrane antigens Indirect immunofluorescence of Mol, LFA-1, and β2-microglobulin was measured by cytometry as described by Todd et al.[3] The channel number (log scale) measuring the mean fluorescence intensity of 5,000 cells that had been exposed to either an experimental or a negative control antibody (anti-Ia) and then to a fluorescein-conjugated goat antimouse immunoglobulin was determined. Logarithmic channel numbers were converted to linear values[27] and the specific mean fluorescence intensity for cells stained by experimental antibodies was calculated by subtracting the mean channel number of cells exposed to the negative control antibody from that of cells stained by the experimental antibodies

RESULTS

Preparation of cytoplasts. To examine the claim that cytoplasts are truly free of granule activity,[1] a comparison of cytoplast membranes with those from intact cells for the appearance of two granule membrane markers was performed. It may be that during cytoplast preparation degranulation occurs and results in the fusion of granule membranes with the plasma membrane. Cytoplasts were prepared according to the method of Roos et al[1] using a Ficoll step gradient containing cytochalasin B. The recoveries of the various soluble enzymes in the isolated fractions are shown in Table 1 The results are similar to those of Roos et al,[1] with the largest amounts of LDH (39%) and protein (42%) being recovered in the cytoplast fractions (band 1). Examination of the material at this position using light microscopy showed only vesicles without nuclei. Since cytoplasts are believed to consist of only cytoplasm surrounded by plasma membrane,[1] the activities of soluble granule enzymes were compared to the cytoplasmic marker LDH as an indication of the relative purity of the cytoplast fraction (Table 2). Comparing the cytoplast fraction to intact PMNs, decreases in all enzyme activities were observed. The largest decreases were in granule constituents, vitamin B_{12} binding protein, β-glucuronidase, myeloperoxidase, and lysozyme, as was expected since cytoplasts have been shown to be deficient in these

Table 1. Marker Analysis of Ficoll Gradient Fractions From Cytoplast Preparations

	Percentage of Recovery*			
Marker	Band 1, Cytoplasts	Band 2, Whole Cells	Band 3, Nuclei and Granules	Total Recovery
Protein	42 ± 5	30 ± 13	34 ± 7	106 ± 11
LDH	39 ± 6	20 ± 3	22 ± 12	81 ± 3
Lysozyme	5 ± 1	16 ± 5	68 ± 15	90 ± 10
Vitamin B_{12}–binding protein	7 ± 4	34 ± 12	51 ± 6	92 ± 22
β-glucuronidase	8 ± 1	23 ± 12	67 ± 18	98 ± 4
Myeloperoxidase	2 ± 1	33 ± 13	63 ± 23	98 ± 26
Alkaline phosphatase	18 ± 1	33 ± 7	36 ± 8	87 ± 3

*Mean percentages (±SD) of activity recovered from fractions obtained during cytoplast preparation The results were obtained from three experiments

Table 2. Activity of Markers From Intact PMNs and the Cytoplast Fraction With Respect to LDH Activity

	Marker/LDH*				
Fraction	Lysozyme × 10^{-3}	Vitamin B_{12}–Binding Protein	β-glucuronidase × 10^{-3}	Myeloperoxidase	Alkaline Phosphatase × 10^2
PMN	1 94	416.0	2.6	5.93	1.0
Cytoplasts	0 24	55.4	0 6	0.37	1.1
	(8×)†	(7.5×)	(4.3×)	(16×)	(0 91×)

*Mean ratio of enzyme activity per unit of LDH activity for three experiments. A unit is defined as μmol/min, except for vitamin B_{12}, which is given in pg.

†Fold decrease in enzyme activity in the cytoplast fraction.

organelles.[1] Alkaline phosphatase activity with respect to LDH did not change.

Degranulation and Mol expression during cytoplast preparation. Several steps during cytoplast preparation such as warming to 37° and treatment with cytochalasin B could cause degranulation and translocation of granule membrane markers. To determine whether degranulation occurs during the early stages of cytoplast preparation, release of granule contents and surface Mol antigen expression were measured. Figure 1 shows a comparison of the release of two granule constituents, lysozyme and vitamin B_{12}–binding protein, and Mol expression of intact cells at each step of cell treatment, then followed by stimulation with A23187. It can be seen that there was an increase in both enzyme release and Mol expression at every condition but the fifth (incubation for an additional 30 minutes in Ficoll with cytochalasin B). During the early preincubation steps of warming the PMNs for five minutes at 37° in Ficoll plus cytochalasin B before layering on the gradients, the largest increase in Mol expression and enzyme release occurred. Subsequent incubation of the PMNs for 30 minutes in Ficoll and cytochalasin B, which would be comparable to the conditions intact cells would be under when they were on the gradient except without centrifugation, did not result in an additional increase in Mol expression However, treatment with A23187 caused a significant increase in Mol expression and enzyme release, indicating that further degranulation was possible.

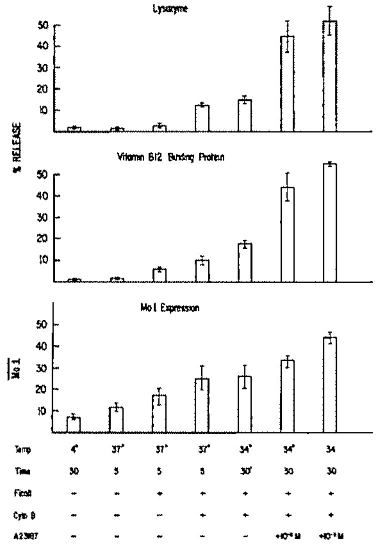

Fig 1. Mol expression and specific granule release from intact PMNs during sequential steps leading up to cytoplast preparation and then followed by activation of intact PMNs with ionophore A23187. Separate aliquots of PMN (5 × 10^7/mL) from the same donor were put on ice or incubated for five minutes at 37 °C, washed, and then treated under the indicated conditions. Specific intensity mean fluorescence arbitrary units (±SE) with anti-Mol antibody and release of enzymes into the cell supernatant (±SE) were measured in four experiments. Cells stimulated with A23187 were incubated for 30 minutes with 12.5% Ficoll plus 20 μmol/L cytochalasin B at 34 °C before exposure to A23187 for ten minutes at 37 °C. The release of LDH was <2% without ionophore and <7% in the presence of ionophore.

Since both Mol and specific granule release increased during cytoplast preparation, the correlation between these two responses was calculated. For Mol expression and vitamin B_{12} binding protein release and for Mol and lysozyme release, the correlation coefficients were .78 and .92 respectively These have a P value of less than .001 for $n = 19$ and, thus, are highly significant. As anticipated, the release of the two granule constituents vitamin B_{12}–binding protein and lysozyme correlated well with each other ($r = .89$, $P < .001$).

Expression of Mol and plasma membrane markers. Although the mean Mol expression increased during the initial stages of cytoplast preparation, it was important to see how Mol behaved in relation to known plasma membrane markers during these manipulations and after cytoplasts had been generated The two plasma membrane markers that were chosen, LFA-1 and β_2m, have been shown to remain constant even after stimulation with f-Met-Leu-Phe.[28,29] Table 3 shows the ratio of Mol to two plasma membrane markers, β_2m and LFA-1, for cells at 4°, after Ficoll plus cytochalasin B treatment and after cytoplast preparation. A comparison of the ratios of Mol to LFA-1 for PMNs incubated in Ficoll with cytochalasin B and for cytoplasts showed that there was an increase (1.9-fold) in the appearance of Mol after cytoplast preparation. Cells left at 4° had a much lower Mol/β_2m ratio than those exposed to Ficoll plus cytochalasin B at 37° (Table 3). This observation confirmed the results shown in Fig 1, namely, that treatment with Ficoll and cytochalasin B caused a significant increase in Mol expression. In close agreement with the Mol/LFA-1 result, the ratio of Mol to β_2m was significantly higher (1.6-fold) for cytoplasts than for PMNs treated with Ficoll plus cytochalasin B. The mean fluorescence intensities for LFA-1 and β_2m on a per-particle basis did not change under any condition (data not shown). Stimulation by A23187 (10^{-6} mol/L) of PMNs treated with Ficoll plus cytochalasin B increased the amount of Mol, indicating that further stores of Mol were available The

Table 3. Ratio of Mol to LFA-1 and to β_2m During the Stages of Cytoplast Preparation

	Ratio of Mean Fluorescence*		
Ratio	PMN	PMN Ficoll + CB†	Cytoplasts
Mol/LFA-1	ND	6.24 ± 0.92	12.0 ± 3.28‡
Mol/β_2m	0.81 ± 0.05	2.65 ± 0.19‡	4.34 ± 1.94‡
Mol/β_2m + A23187 (10^{-6} mol/L)	ND	4.3 ± 0.56§	4.36 ± 2.8

Abbreviations: CB, cytochalasin B, ND, not determined

*Ratio of mean fluorescence (±SD) of Mol to either of two plasma membrane markers from three experiments

†PMN were incubated at 37 °C in Ficoll with 20 μmol/L cytochalasin B for 30 minutes during cytoplast preparation.

‡$P < .01$ (using paired statistics) when compared to values for preceding cell treatment.

§$P < .01$ (using paired statistics) when compared to Ficoll + cytochalasin B–treated cells without A23187.

ratio for cytoplasts did not change after stimulation with A23187, which is consistent with the notion that the cytoplasts were deficient in granules.

Analysis of cytochrome b in plasma membrane preparations. Another specific granule marker, cytochrome b, was followed during cytoplast preparation as a monitor of degranulation. Since we wished to measure the activity of cytochrome b that had translocated to the plasma membrane and not that which was still present in the granules, analysis of cytochrome b was performed on isolated plasma membrane fractions. Table 4 shows a comparison of the specific activities of cytochrome b and plasma membrane markers in untreated PMNs, PMNs treated with Ficoll plus cytochalasin B, and cytoplasts. There was a significant increase in the specific activity of cytochrome b in plasma membranes from cells that had been treated with Ficoll plus cytochalasin B as well as those from cytoplast preparations. To determine whether Ficoll plus cytochalasin B increased the amount of cytochrome b in cells, the total cytochrome b content of intact cells with and without this treatment was measured; no difference in cytochrome b content was found (data not shown).

The activities of alkaline phosphatase and Mg-ATPase were also measured (Table 4) so that cytochrome b could be compared to two membrane markers in a manner analogous to the comparison of Mol to LFA-1 and to β_2m (Table 3). This comparison was crucial since the relative protein content of plasma membranes isolated from PMNs appeared to decrease after treatment with Ficoll plus cytochalasin B and, again, after cytoplast preparation. The activity of alkaline phosphatase also appeared to decrease, making the usefulness of this marker questionable. Another plasma membrane marker, Mg-ATPase, did not change during these treatments. Table 5 shows the ratios of cytochrome b content relative to the two plasma membrane marker activities. As predicted from the earlier results with Mol expression, the ratio of cytochrome b to alkaline phosphatase was significantly higher in plasma membranes from cells treated with Ficoll plus cytochalasin B than those from untreated PMN. A similar significant increase was found for the cytochrome b/Mg-ATPase ratio. The preparation of cytoplasts further increased the ratio of cytochrome b to alkaline phosphatase; the ratio of cytochrome b to Mg-ATPase was not altered by this final step.

Table 4. Enzyme Activity in Plasma Membrane Preparations Obtained at Different Stages of Cytoplast Preparation

	Specific Activity*		
Marker	PMN	PMN Ficoll + CB†	Cytoplasts
Cytochrome b (× 10^7)	1.4 ± 0.06	2.82 ± 0.1‡	3.87 ± 0.6‡
Alkaline phosphatase (× 10^2)	11.6 ± 4.0	6.77 ± 1.14	2.4 ± 0.15
Mg-ATPase (× 10^5)	1.76 ± 0.7	2.08 ± 0.41	2.64 ± 0.21

*Mean specific activity was measured in terms of (μmol/mg protein) for cytochrome b and (μmol/min/mg protein) for plasma membrane enzymes (±SE) for at least three experiments. Marker descriptions include the exponents for the specific activities

†PMNs were incubated at 34° in Ficoll with 20 μmol/L cytochalasin B for 30 minutes during cytoplast preparation.

‡$P < .01$ as an indicator of significance when compared to the preceding stage.

DISCUSSION

The question of whether cytoplasts are devoid of all granule activities has been considered. Our preparation of cytoplasts was similar to that of Roos et al[1] in recovery of protein and marker enzyme activities. Our yield of cytoplast material was slightly lower than that reported by the latter investigators; recovery of LDH was 38% instead of 48% and that of alkaline phosphatase was 18% instead of 33%. In spite

Table 5. Ratio of Cytochrome b Activity to Two Plasma Membrane Enzymes Obtained at Different Stages of Cytoplast Preparation

	Ratio of Cytochrome b to Membrane Enzyme*		
Plasma Membrane Enzyme	PMN	PMN Ficoll + CB†	Cytoplasts
Alkaline phosphatase (× 10^5)	1.23 ± 0.4	4.2 ± 0.07‡	16.1 ± 1.3‡
Mg-ATPase (× 10^5)	5.85 ± 0.09	16.0 ± 2.8‡	14.6 ± 1.0

*Ratio of the mean specific activity of markers from at least three experiments (±SD)

†PMNs were incubated at 34° in Ficoll with 20 μmol/L cytochalasin B for 30 minutes during cytoplast preparation.

‡$P < .01$ as an indicator of significance when compared to the preceding stage.

of this, our cytoplast preparation was similar in having greatly reduced amounts of soluble granule enzymes.

Our studies indicate that degranulation and translocation of two granule membrane markers occurred during cytoplast preparation. In particular, we showed that the series of manipulations involved in cytoplast preparation increased Mol expression and the release of lysozyme and vitamin B_{12}–binding protein in a stepwise fashion. The good correlation between the release of the two specific granule constituents and Mol expression provided further evidence that Mol was a marker of degranulation. Significant increases in the ratio of Mol/β_2m (3.3-fold, Table 3) as well as that of cytochrome b/Mg-ATPase in the cell membrane (2.7-fold, Table 5) occurred upon incubation of PMNs with Ficoll plus 20 μmol/L cytochalasin B. The preparation of cytoplasts further increased the appearance of Mol on the cell surface (1.6-fold, Table 3) and the specific activity of cytochrome b in the plasma membrane (1.4-fold, Table 4). Therefore, our data suggest that cytoplast membranes contain significantly higher amounts of granule membrane activity than those of untreated cells.

Degranulation was shown to occur during the Ficoll and cytochalasin B pretreatment of cells for cytoplast preparation. The demonstration that Mol expression increased in a fashion similar to the release of lysozyme and vitamin B_{12}–binding protein is an important finding. Although degranulation had previously been shown to occur under conditions in which Mol expression is increased,[3] this is the first report of a good correlation between Mol expression and enzyme release. This is not to be expected since Mol is a surface antigen that, unlike granule enzymes, has a low but significant level of expression on unstimulated cells (Fig 1). It should be noted that this good correlation does not necessarily support the hypothesis that Mol is located in specific granules.

The largest increase in Mol expression as compared to PMNs left at 4° occurred during the five-minute incubation of cells with Ficoll and cytochalasin B at 37°. The ability of cytochalasin B to enhance PMN responses is well documented.[10,30–33] Cytochalasin B is thought to enhance stimulation by increasing influxes of Ca^{+2} and Na^{+} ions.[32] There is one report showing that cytochalasin B alone causes the stimulation of oxidase activity.[33] Our data also indicate that cytochalasin B can cause a significant amount of degranulation in the presence of Ficoll.

Stimulation with the calcium ionophore A23187 in the presence of cytochalasin B increased the amount of Mol expressed and the amount of granule enzymes released from intact cells, but not from cytoplasts. The inability of A23187 to increase Mol in cytoplasts indicates that internal stores of Mol were not present in cytoplasts. This finding is in agreement with that of Lutter et al who showed that stimulation with PMA increased the content of cytochrome b in the membranes of intact cells but not those of cytoplasts.[9]

It is noteworthy that stimulation with A23187 did not increase Mol by the same proportion as it did the release of lysozyme and vitamin B_{12}–binding protein. This could to some extent be attributed to higher baseline levels of Mol, which, as already discussed, could be increased by low-level degranulation during the cell's history. The higher stimulated levels of lysozyme as compared to Mol might be explained by the contributions of enzyme from azurophil granules. That lysozyme might also be released from this other source was suggested by the release of β-glucuronidase, an azurophil granule enzyme, after stimulation by A23187 (data not shown).

The comparison of Mol to two membrane markers, LFA-1 and β_2m, demonstrated that the preparation of cytoplasts caused further translocation of Mol to the cell surface. The amounts of the two plasma membrane markers have been shown not to change after exposure to degranulating stimuli,[28,29] making them suitable steady-state determinants for comparison to Mol. The higher ratio of Mol/β_2m for cells treated with Ficoll plus cytochalasin B as compared to untreated PMNs (Table 3) is consistent with the data in Fig 1 correlating the mean fluorescence of Mol to enzyme release. The relationship between Mol and the plasma membrane markers LFA-1 and β_2m was analogous to the positive correlation between the specific activity of cytochrome b with respect to Mg-ATPase (which was also found to increase under similar conditions).

To determine whether another specific granule marker, cytochrome b, also translocated to the cell membrane during cytoplast preparation, plasma membrane fractions were prepared. These fractions were enriched 12-fold in alkaline phosphatase and Mg-ATPase activities (data not shown). The plasma membrane fractions from cytoplast gradients had a smaller increase (sixfold) in specific activity, presumably because with most of the granules eliminated the plasma membrane protein could make a larger contribution to the total. As with Mol expression, the largest increase (twofold) in the specific activity of cytochrome b occurred when the cells were incubated in the presence of Ficoll with cytochalasin B; cytoplast preparation further increased its specific activity (1.4-fold). When the cytochrome b content was compared to Mg-ATPase activity, a significant increase in the ratio occurred upon incubation of cells in Ficoll with cytochalasin B. The comparison of cytochrome b to alkaline phosphatase activity magnified these differences, presumably because of the loss of enzyme activity with each treatment.

Our results differ somewhat from some other reports measuring cytochrome b content in cytoplasts as compared to intact cells.[9,10] Some investigators used untreated, intact PMNs as controls, whereas we examined both these as well as cells treated with Ficoll plus cytochalasin B. Analyzing the specific activity of cytochrome b with respect to protein, Ohno et al[10] found no difference between the plasma membranes of intact cells and those of cytoplasts. In contrast, we demonstrated significant differences in cytochrome b activity among plasma membrane preparations from untreated cells, from cells treated with Ficoll plus cytochalasin B, and from cytoplasts. Since granule membranes have a density similar to plasma membranes,[7] there may be a problem of contamination of plasma membrane fractions. In another report, Lutter et al compared cytochrome b to alkaline phosphatase activity and found that plasma membranes of intact cells had a ratio twice as high as that of cytoplast membranes.[9] Our results showed the opposite, with plasma membranes of cytoplasts having a higher cytochrome b/alkaline phosphatase ratio than those of either PMNs treated with Ficoll plus cytochalasin B or untreated cells. Contributing to these results are the large changes in alkaline phosphatase specific activity between the different fractions (Table 4). This could be due to differences in protein composition of the plasma membrane induced by the various treatments. However, this decrease in specific activity during cytoplast preparations was not seen with Mg-ATPase, indicating that the two plasma membrane markers were not distributing identically. Nonetheless, no matter how cytochrome b specific activity was calculated (eg, with respect to protein, alkaline phosphatase, or Mg-ATPase), a significant increase in activity was found after treatment with Ficoll plus cytochalasin B. Furthermore, this is in agreement with the results of Mol expression (Table 3). Thus, these data strongly suggest enrichment of granule membrane components in the plasma membrane during the steps of cytoplast preparation.

The intracellular sources of Mol and cytochrome b have not been determined. Mol has been shown to have an intracellular pool that migrates to the plasma membrane after activation of human PMNs.[3,34] One source for Mol that has been implicated by some recent experiments is the tertiary granule, a gelatinase-containing granule originally described by Dewald et al.[35] We have shown that by using stimuli under conditions in which specific and azurophilic granules are not released, both Mol expression and gelatinase release occur.[36] We infer from these results that one intracellular source of Mol is the tertiary granule. One source of evidence locating cytochrome b to the specific granule comes from studies with a specific granule–deficient patient.[37] The PMNs from this patient, which lack specific granules, do not contain cytochrome b in any granule fraction, implying that the specific granule is the source of cytochrome b. On the other hand, PMNs from this patient are capable of up-regulating Mol (unpublished data), indicating that other sources for Mol exist besides the specific granule. Another possible intracellular membrane source for these markers, which has not been excluded by these experiments, may be the receptor recycling system, receptosomes.[38]

In conclusion, degranulation occurred during the various stages of PMN cytoplast preparation as indicated by enzyme release and the translocation of granule membrane markers to the PMN plasma membrane. The increases in Mol expression and cytochrome b activity on the cell membrane upon incubation in Ficoll plus cytochalasin B and during cytoplast preparation indicate that cytoplasts are not devoid of granule membrane content.

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Evaluation by Quantitative Acid Elution and Radioimmunoassay of Multiple Classes of Immunoglobulins and Serum Albumin Associated With Platelets in Idiopathic Thrombocytopenic Purpura

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Immunoglobulins (Igs) and serum albumin were eluted from normal platelets and platelets from patients with idiopathic thrombocytopenic purpura (ITP) with a quantitative acid elution procedure followed by solid-phase radioimmunoassay (SPRIA). Acid elution was shown to release a reproducible fraction of platelet-associated Igs, and the amounts released per platelet were independent of the platelet concentration over a wide range of concentrations. This procedure is suitable for sensitive, reproducible, and specific quantitation of large numbers of samples. Washed platelets from 13 normal donors contained the following components (expressed in femtograms per platelet, mean ± 2 SEM): IgG, 1.40 ± 0.26; IgA, 0.72 ± 0.36; IgM 0.078 ± 0.036; albumin 7.7 ± 1.5. Immunoglobulins and albumin eluted from the platelets of ten ITP patients (two in remission), expressed as femtograms per platelet, mean (range), were: IgG 104 (0.3 to 750); IgA 90 (0.9 to 715); IgM 162 (1.2 to 1,300); and albumin 34 (6.8 to 199). All platelet-associated Igs from thrombocytopenic ITP patients were found to be elevated twofold to 2,300-fold with one Ig class occasionally elevated 50-fold to 100-fold higher than the others. A similar group of ten thrombocytopenic ITP patients was found to have twofold to 26-fold elevations of platelet-associated albumin. This demonstration of increases in multiple classes of Igs as well as serum albumin associated with platelets from ITP patients suggests that some nonimmune process may be contributing to the phenomenon of increased platelet-associated proteins in ITP.

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ITP FACTOR capable of inducing thrombocytopenia by passive transfer has been shown to be present in the purified IgG fraction from the plasma of patients with idiopathic thrombocytopenic purpura (ITP).[1,2] Measurement of platelet-associated IgG (PAIgG) has become a frequently used diagnostic test in ITP. Numerous reports have shown that PAIgG is elevated in most cases of ITP and that the degree of elevation is inversely related to the platelet count (for review, see ref. 3). Recent reports, however, have described some ITP patients who have elevated platelet-associated IgM[4-9]; other studies have shown increased platelet-associated IgA as well.[9-12] Finally, elevations of platelet-associated C3[9,13,25] and C4[9] in thrombocytopenic ITP patients have been reported.

We have found that washed platelets from normal individuals have measurable levels of both IgA and IgM in addition to IgG; furthermore, normal platelets also possess measurable quantities of albumin. Our findings indicate that when PAIgG is elevated in ITP, platelet-associated IgM, IgA, and albumin are also elevated in most instances. These results suggest that the increased levels of immunoglobulins in platelet preparations from ITP patients may be due partly to mechanisms unrelated to antigen–antibody binding; the same mechanisms may also account for the attachment of albumin and possibly other serum proteins.

MATERIALS AND METHODS

Platelet preparation. Blood was drawn by the two-syringe technique and immediately added to a 10% solution of dipotassium EDTA 17 µl/mL of whole blood. The platelet-rich plasma (PRP) was made by centrifuging whole blood at 400 g for four minutes. PRP was centrifuged at 1,500 g for 15 minutes, and the platelet pellet was suspended in 1.0% ammonium oxalate for ten minutes. After being pelleted, the platelets were washed three times in 0.15 mol/L of NaCl (saline) and suspended in saline. Platelets were either eluted immediately or were stored at 4 °C with 0.02% sodium azide.

Quantitative acid elution. All subsequent steps were carried out at 4 °C unless otherwise noted. The general elution procedure was to suspend the platelets in unbuffered saline and add some volume of acid buffer with bovine serum albumin (BSA) (Miles, Naperville, Ill, Fraction V) to achieve a final acid concentration of ~50 mmol/L and a final BSA concentration of 0.5%; for example, a 200-µl aliquot of the final saline suspension containing from 0.1 to 1 × 10^8 platelets was acidified by the addition of 100 µL of 150 mmol/L of H_3PO_4, 100 mmol/L of NaCl, 1.5% BSA, pH 2.8. Acetic acid (150 mmol/L), titrated to pH 2.8 with HCl, can be used in place of H_3PO_4 with identical results. After ten minutes, the suspension was centrifuged in a Beckman Microfuge 12 at maximum speed (~10,000 g) for two minutes. A predetermined amount of 2.5 mol/L of Tris base was added to 250 µL of the supernatant to raise the pH to 8.0 to 8.1, and sodium azide was added to 0.02%. This platelet extract could be stored at 4 °C for periods up to two months without adsorption of the Igs to polyethylene tubes or loss of immunologic reactivity.

Ig quantitation. Igs were quantitated by a solid-phase radioimmunoassay (SPRIA) technique using flexible polyvinyl chloride plates (Dynatech, Alexandria, Va) coated with heavy chain-specific, affinity-purified, goat anti-human IgG, IgA, or IgM, (Kirkegaard & Perry Labs, Rockville, Md). The plates were coated by adding 80 µl of 10 mmol/L of $NaPO_4$, pH 8.0, with 0.02% NaN_3 to each well of the 96-well microtiter plate followed by 20 µL per well of the heavy chain-specific antibody, at 25 ng/µL, in the same phosphate buffer. To avoid adsorption of the anti-Ig to the test tube, all procedures were done at 4 °C, and only enough of the antibody was diluted from a 1 mg/mL solution in 150 mmol/L of NaCl, 10 mmol/L PO_4, pH 7.4 (PBS) for preparation of one plate. The plates were incubated overnight at 4 °C in a humidified airtight container and were then washed three times by flooding with phosphate-buffered saline (PBS), 0.5% BSA, 0.02% NaN_3 (BSA/PBS) and incubated with BSA/PBS for at least 30 minutes prior to use to block residual protein binding sites. Usually, 10 to 12 plates were prepared at one time; these could be stored full of the final wash solution at 4 °C in a humidified chamber for as long as three months.

The same heavy chain-specific antibody used to coat the plates

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was used as the labeled second antibody; ^{125}I was coupled to these antibodies by the chloramine-T method. Na^{125}I [16.6 mCi/μg iodine (Amersham, Arlington Heights, Ill, #IMS300)] was added to the antibody in the ratio of 2 μCi/mg of protein in 100 mmol/L (final) of phosphate buffer, pH 7.5. The reaction was initiated by the addition of chloramine-T (0 5 μg/μg of protein), incubated with gentle mixing for 90 seconds and terminated by the addition of a saturated tyrosine solution (0.5 mL/μg of protein). After 60 seconds, the mixture was applied to a Dowex AG1-X8 (200 to 400 mesh) column (7 × 25mm) which was preequilibrated with borate (345 mmol/L)-buffered BSA (0.1%) solution (BBSA, pH 8.55); the column was washed with successive 500-μL aliquots of BBSA. The major portion of radioactivity eluted in the second and third column fractions. More than 95% of the radioactivity in this preparation was eluted in the void volume of a Sephadex G25 column (Pharmacia PG-10) Therefore, the eluate from the Dowex column was used without further purification.

Between 10 and 40 μL of the platelet acid eluates were added to antibody-coated microtiter wells that had been sucked dry of the BSA/PBS solution and filled with 100 μL of radioimmunoassay (RIA) buffer (100 mmol/L of Tris, pH 8.1, 50 mmol/L of NaCl, 0.5% BSA, 0.02% NaN_3). Chromatographically purified IgG (Cappel), secretory IgA (Calbiochem, Malvern, Pa), or IgM (Calbiochem, San Diego, Calif) standards from 0.1 to 500 ng per well, in the same volume as the eluates, were added to other wells for the standard curves, in a buffer that reproduced the neutralized acid eluate. The plates were either incubated at room temperature for two hours or overnight at 4 °C. The plate was then placed on an ice bed for at least ten min, the unbound material was thrown off, and the plate was washed three times by flooding with ice-cold BSA/PBS. The wells were aspirated dry, and 100 μL of ^{125}I-labeled goat anti-human Ig (50,000 cpm per well) in RIA buffer was added to each well and incubated for 90 minutes at 21 °C. The plates were then chilled on ice for ten minutes, sucked dry, and washed three times with 160 μL of cold BSA/PBS. The plates were dried, and individual wells were cut out and counted in a gamma counter. Maximum binding was achieved with 500 ng per well of antigen (generally between 9,000 cpm and 15,000 cpm) The Ig blank (usually <200 cpm) was subtracted from all data.

The standard curves were graphed as the logit (+5) of cpm *v* the negative log of Ig which allows a least-squares linear regression to be fit to the data (Fig 1, insert); reliable data were obtained in the range of 0.5 to 30 ng of Ig per well.

Serum albumin quantitation. Human serum albumin (HSA) was quantitated by a solid-phase competitive binding assay. One hundred microliters of diluted goat antiserum to HSA (Cappel) was added to the microtiter well (500 μg of protein per well, whole serum) and the plates were incubated, washed, and blocked as with the Ig assays. Crystalline HSA (Miles, Naperville, Ill) was labeled, as described above, with ^{125}I, 4 μCi/μg of protein; the labeled serum albumin was mixed with the platelet eluate or an albumin standard, and 100 μL of the mixture containing 60,000 cpm was added to the antiserum-coated wells, incubated for two hours at 21 °C or overnight at 4 °C, washed three times as with the Ig assays, dried, and counted. The maximum binding was typically 10,000 cpm, which could be maximally displaced by 500 ng of HSA per well; the useful range of the assay was typically from 2 to 200 ng per well This assay system could therefore measure platelet-associated albumin (PAA) given as little as 5 mL of whole blood from a normal donor or 40 mL of whole blood from a severely thrombocytopenic patient (a platelet count of 10,000/μL).

Patients with ITP All patients had thrombocytopenia with an increased number of megakaryocytes in the bone marrow and no associated abnormality or disease process that might account for thrombocytopenia. The patients' tests for antinuclear antibodies and rheumatoid factor were all negative. The one pediatric patient studied had acute ITP, and all others had chronic ITP Platelet counts of the patients are tabulated in the text.

RESULTS

Sensitivity of these assays was of primary importance in evaluating samples with scarce material from severely thrombocytopenic patients. Figure 1 shows that this solid-phase sandwich system is accurate to <1ng/40 μL. When necessary, the sensitivity of these assays could be increased at least tenfold by using a smaller volume of the diluting RIA buffer and/or by concentrating the platelets in the acid elution procedure. Dilution of sample in the RIA buffer was performed to prevent minor pH variations between empirically neutralized samples. The use of polyclonal antibodies gives a wide standard curve which spans nearly two logs of concentration. The logit transformation of the standard curve, plotted by linear regression analysis, is convenient for measuring unknowns.

The ability of the assay system to distinguish between the classes of immunoglobulin was particularly critical for this study. The heavy chain-specific antibodies show insignificant cross-reactivity with other classes for the purposes of this study (Fig 1). The three other combinations for testing

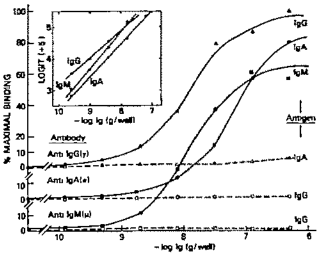

Fig 1. Binding curves for IgG on anti-IgG (▲——▲), IgA on anti-IgG (△—△), IgA on anti-IgA (●——●), IgG on anti-IgA (O—O), IgM on anti-IgM (■——■), and IgG on anti-IgM (□—□). Inset: Logit was defined as $\text{Log}_{10}P/(1 - P)$ where P equals the fraction of maximal specific binding. Typical logit transformation standard curves for IgG (△), IgA (O), and IgM (□), run in duplicate, are shown. The average coefficient of variation was 0.36 ± 0.12 for (logit +5) values between 3 and 5.

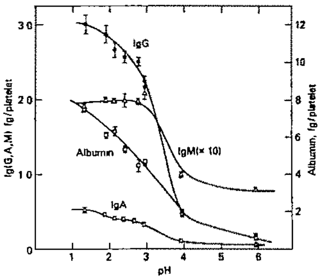

Fig 2. pH-dependent elution of gamma globulins and albumin: IgG (●), IgA (O), IgM (△), and albumin (□) eluted per platelet. Values are shown as mean ± SEM of duplicate assays on pooled platelets of three normal individuals. Note that IgM values are multiplied by 10 in this plot. The acid buffer was 50 mmol/L of PO_4, 50 mmol/L of acetic acid, 50 mmol/L NaCl, 0.5% bovine serum albumin (BSA), and 0.02% NaN_3 for each point; pH was measured at room temperature.

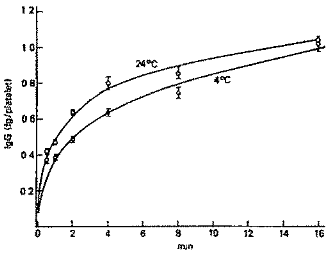

Fig 3. Time course of IgG elution at pH 2.65; 2 × 10^8 platelets in 200 µL saline were acidified with 100 µL of acid buffer.

cross-reactivity (anti-IgG with IgM, anti-IgA with IgM, and anti-IgM with IgA) show similar results.

The effect of pH on the elution of various platelet-associated proteins is shown in Fig 2. IgG and IgM show a sharp increase in elution below pH 4, IgA shows a more gradual increase, and albumin shows a progressive elution over a wide range of decreasing pH values. A pH between 2.7 and 2.8 was suitable for eluting a high percentage of Igs at minimal risk of denaturation Treatment of IgG, IgM, IgA, and HSA standard proteins under conditions of acid elution produced no detectable change of antigenicity as judged by comparing assays before and after treatment. Moreover, when 5 to 240 ng/µL of IgG, IgM, IgA, or HSA was added to 2 × 10^8 platelets in 200 µL before acid elution was performed, essentially 100% of the added proteins was recovered, as measured by the solid-phase RIA (SPRIA) technique. Thus, acid treatment per se and the presence of soluble platelet materials released by acid treatment did not interfere with the specificity or sensitivity of the SPRIA.

The time course of elution of IgG from washed platelets is shown in Fig 3. The amount of IgG eluted approached an asymptotic value after eight minutes, and the rate of elution was only slightly increased at 24 °C as compared to 4 °C. A convenient elution condition was ten minutes at 4 °C.

A linear relationship should exist between platelet concentration and Ig yield. Because high concentrations of platelets could buffer the acid and alter Ig yield, the effect of platelet concentration on the linearity of the Ig elution was evaluated. Figure 4 shows a linear relationship between IgG, IgA, and IgM and the number of platelets up to 2 × 10^8 platelets/700 µL of acidified platelet suspension.

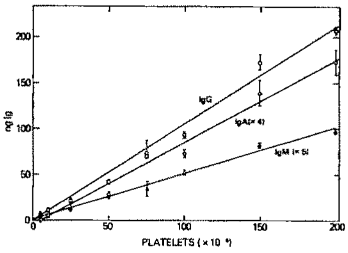

Fig 4. Linearity of Ig elution. Duplicate platelet samples were suspended in 200 µL of saline to which was added 500 µL of 50 mmol/L of H_3PO_4, 0.5% bovine serum albumin (BSA), pH 2.8. IgG (□), IgA (○), and IgM (△).

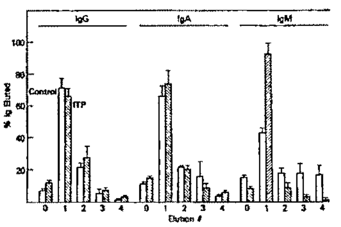

Fig 5. Multiple elutions of Igs from pooled platelets of three normal individuals (open bars) and four idiopathic thrombocytopenic purpura (ITP) patients (hatched bars) in duplicate. Error bars are 1 SEM. Zero elution refers to the Ig present in the supernatant of the centrifuged saline suspension that was never acidified, and the value represents percentage of total eluted material.

A single elution was used for most experiments. However, this did not appear to release 100% of the Igs from the platelets (Fig 5). As judged by summation of four sequential elutions, the first elution released ~70% of platelet-associated IgG and IgA from both control and ITP platelets. IgM release in the first elution of control platelets was ~43% whereas that of ITP platelets was ~90% in the first elution. The variation in the yield of Igs from the first elution is ≤10%. Because IgM levels were quite small and near the limits of sensitivity of the assay, there was some added uncertainty in the percentages of total IgM in elutions of control platelets. Platelets were also disrupted by sonication of freeze-thawing and were then acid-eluted and centrifuged at 20,000 g for 30 minutes; this increased the single elution yield of IgG, IgA, and IgM between 50% and 100%.

The amounts of Igs associated with normal platelets and ITP platelets are shown in Fig 6. Average levels of IgG, IgA, and IgM, from a sample of 13 normal donors are 1.4 ± 0.13, 0.72 ± 0.18, and 0.078 ± 0.018 fg per platelet, respectively (mean ± SEM). The ratio of normal value of PAIgA to PAIgG (0.5) appears to be higher than the ratio of average plasma levels (0.17), whereas the ratio of normal value of PAIgM to PAIgG (0.056) is close to that seen in plasma (0.074). Similarly, the ratio of PAIgG to platelet albumin (0.25) is not different from the ratio normally found in serum (0.26).

The mean platelet-associated Ig levels were elevated for all three Ig classes in ITP patients. In this series of eight patients (two were tested twice with at least one month between samples), IgG was elevated 70-fold; IgA was elevated 125-fold, and IgM was elevated 2,080-fold. The platelet counts for the ITP patients were, from bottom to top in the IgG

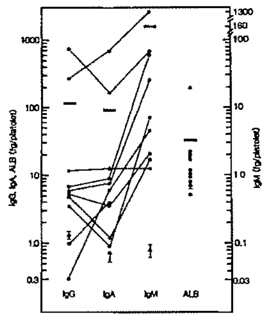

Fig 6. Platelet-associated serum proteins in normal donors and patients with idiopathic thrombocytopenia purpura (ITP). Individual values for ITP patients (●); means for this group are solid horizontal bars. Triangles are the mean ± SEM for 13 normal donors. Lines connect values obtained on a single platelet sample.

column (× $10^3/\mu L$: A, 215; B, 200; C, 27; D, 6; E, 9; F, 21; G, 45; H, 6; I, 2; and J, 5). Samples A and H and samples C and I were taken from the same individuals. Samples A and B were taken from ITP patients in remission. PRP and platelets from a saline wash of the initial red cell fraction were pooled for sample J. Platelet-associated human serum albumin (PAA) was also measured in a different group of ten ITP patients and 12 controls; it was elevated: normal, 7.7 ± 1.5 fg per platelet; ITP, 33 ± 18 fg per platelet (mean ± SEM). These PAA levels are from a different group of patients than that used for the Ig study and did not include two of the patients with the highest Ig levels. Therefore, the PAA data are not strictly comparable to the PAIg measurements except that the mean values of all measured platelet-associated serum proteins were elevated in ITP patients. Platelet counts of ITP patients on whom PAA levels were obtained were, from bottom to top in the ALB column (× $10^3/\mu L$: 73, 50, 30, 18, 80, 54, 10, 21, 27, and 4).

There were two examples of normal PAIgG with elevated PAIgA or PAIgM and one instance of normal PAIgA and elevated PAIgG and PAIgM. Linear regression analysis was made to determine if there was a correlation between the levels of different Ig classes. There was no significant correlation between IgG and IgA ($r = 0.47$, $n = 10$) or IgG and IgM ($r = 0.24$, $n = 9$); however, there was a significant correlation between IgA and IgM ($r = 0.95$, $n = 9$, $P > .01$). This latter correlation disappears if the patient with the highest Ig levels, a pediatric ITP patient, is removed from the group ($r = 0.31$, $n = 8$). Finally, there is high variability in the patient groups: (mean ± SEM) IgG 104 ± 74; IgA 90 ± 70; and IgM 162 ± 142 fg per platelet.

DISCUSSION

Others have used SPRIA or enzyme-linked procedures for the measurement of platelet-associated IgG.[9,14–16] The present modification, using an antibody-antigen-antibody procedure to assay soluble and eluted platelet-associated proteins permits efficient binding of the antigen of interest to the microtiter plate regardless of the concentration of other proteins in the sample. This is in contrast to procedures in which the antigen is bound directly to the microtiter plate, in which case the concentration of the antigen of interest must be relatively high for efficient quantitation because all proteins in cell extracts and eluates or serum samples compete for binding to a degree approximately proportional to their concentration. The SPRIA described is therefore more sensitive than previously described assays for measuring specific proteins in mixture with other proteins. Another advantage of the acid elution SPRIA procedure, in contrast to measurement using monoclonal anti-IgG or protein A binding to intact platelets,[17–19] is the ability, with no ambiguity, to relate the eluted, soluble Ig to a purified, soluble reference Ig by a standard SPRIA curve. This eliminates assumptions about the availability, affinity, and stoichiometry of binding sites of platelet membrane-associated IgG molecules for assay reagents. Reports using procedures in which such assumptions are used in final calculations[18,19] gave normal PAIgG values 15 to 30 times lower than that determined in this study and most others (see ref. 3, p 315).

The nature of the antibody preparation has significant effects on the SPRIA technique described. Microtiter plates coated with affinity-purified antibodies have a much higher capacity and potentially greater selectivity than does the equivalent antiserum or even purified IgG fraction. Polyclonal antibodies, because of their heterogeneity, generally give binding curves which reliably span up to 2 logs of antigen concentration, whereas monoclonal antibodies have much steeper antigen binding curves. The latter situation may require a larger number of dilutions to be certain that unknowns fall on a reliable portion of the standard curve.

Selectivity of the antibodies used in this report is also a significant point. Because at least one report specifically denied the presence of IgA and IgM on normal platelets,[5] it was necessary to demonstrate that the antibodies used in this procedure were sufficiently specific to avoid spurious results. The cross-reactivity of each Ig assay system for other Ig classes was negligible.

The acid elution of platelet-associated proteins is a convenient nondenaturing procedure that is much less disruptive of platelets and their membranes than either detergent[20] or freeze-thaw sonication.[5] Platelets treated with detergent become nonrecognizable as cells, and remaining insoluble material appears as debris. Although sonication treatment is difficult to compare among laboratories, this treatment usually results in total mechanical disruption of platelet membranes and solubilization of a substantial amount of intracellular material. Acid elution at pH 2.8 leaves platelets morphologically distinguishable by electron microscopy and does not produce the fragmentation and debris formation that is characteristic of the more disruptive procedures. The percentage of recovery of total platelet-associated proteins from the acid elution is reproducible and appears to be identical for normal and ITP platelets with respect to IgG and IgA. Although the pattern of recovery for IgM appears to be different for normal and ITP platelets, this could reflect the small quantities of IgM released from control platelets relative to the sensitivity of the assay, particularly in eluates subsequent to the first. Whether or not the SPRIA we used measures only surface proteins on platelets is open to question. We did measure PAIgG of 20 normal controls using the antiglobulin consumption assay[21] with the same antiglobulin reagents and standard IgG used in the SPRIA. The mean value for normal PAIgG was 1.9 ± 1.5 (2 SD) fg per platelet as compared with the mean SPRIA value of 1.4 fg per platelet. Because the antiglobulin consumption assay and the SPRIA measure similar amounts of PAIgG, the assumption by some workers that the former test measures only surface IgG may also apply to the SPRIA, even though the true value for surface PAIgG remains controversial.[19] However, we do not claim to measure only surface proteins by the SPRIA but have shown that the proteins measured are soluble, antigenically intact, and reproducibly measurable by an unambiguous quantitative assay.

This investigation demonstrates two main points. First, it has been shown that there are measurable levels of IgA, IgM, and albumin, as well as of IgG, on normal platelets. Second, all three immunoglobulin classes associated with platelets and even platelet-associated albumin were elevated in most of the ITP patients studied.

Measurement of PAIgG is a frequently used diagnostic test in ITP. Elevations of PAIgG are often interpreted as the amounts of specific antiplatelet antibody bound to platelets and responsible for their destruction. However, we[3,22] and others[23,24] have questioned this interpretation for several reasons. The increased quantities of PAIgG involved in ITP are higher by one to several orders of magnitude than cellular antibody levels causing platelet destruction in well-defined disorders of immunologic origin involving isoantibodies or drug antibodies,[3] and are often more than tenfold the amounts of these antibodies that saturate platelets in vitro.[3] If the high levels of immunoglobulin associated with platelets in ITP are responsible for platelet destruction, the mechanism of destruction must be significantly different from that caused by high-affinity antiplatelet isoantibodies or drug antibodies. Moreover, highly elevated PAIgG levels similar to those found in ITP have also been associated with thrombocytopenias of apparent nonimmune origin.[23] Other major problems of interpretation are raised by the lack of correlation between direct PAIgG measurements in ITP and indirect serologic assays for antiplatelet antibodies, even when such assays are performed in the same test system used for measuring the direct PAIgG,[22,25] and by the finding of elevated albumin levels associated with platelets from ITP patients.[26,27] Finally, the demonstration of rapid increases in platelet-associated dog IgG and dog albumin when dogs are made acutely thrombocytopenic by the infusion of rabbit anti-dog platelet sera[26] further supports the consideration that elevations of PAIgG in ITP, may not reflect the actual amounts of specific antiplatelet antibody responsible for platelet injury and destruction. Antibodies responsible for ITP may destroy platelets at the same low levels per platelet as do isoantibodies or drug antibodies that are not detectable by direct assays on circulating platelets. Platelet destruction may then lead to high elevations of platelet-associated proteins by a secondary nonspecific phenomenon.

One postulated mechanism for elevating PAIgG is promotion of accessibility of platelet membrane Fc receptors by injurious specific ITP antibody, leading to nonspecific immunoglobulin binding.[28] Certain aspects of nonspecific binding of IgG, and presumably other Igs, however, remain unclear. Although a platelet Fc receptor of IgG has been characterized[29] and quantitated,[30] it has not been proven that this type of binding can account for the relatively large amount of IgG associated with extensively washed normal platelets (~100-fold that for a normal red cell) or the many-fold increases in PAIgG that may accompany ITP since the IgG "specifically" bound by Fc receptor was reported to be removed by a single PBS wash.[30] Moreover, Fc binding does not account for elevated platelet-associated albumin

We have proposed an alternate mechanism for elevation of platelet-associated immunoglobulin and albumin that appears to be as reasonable as other theories to explain the observed phenomena; namely, that entrapment of plasma components in the platelet canalicular system or in resealed cell membrane fragments may account for the large amounts of plasma proteins associated with platelets normally and in pathological states when fragmentation is apparently augmented by platelet destruction.[22] Growing evidence shows that a component of the increase in immunoglobulins associated with platelets in ITP involves a nonspecific process whether by this postulated "trapping" mechanism or other mechanisms. The size of the nonspecific component and the exact mechanism of the phenomenon await further studies.

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Reevaluation of Cytochrome b and Flavin Adenine Dinucleotide in Neutrophils From Patients With Chronic Granulomatous Disease and Description of a Family With Probable Autosomal Recessive Inheritance of Cytochrome b Deficiency

By Yohichiroh Ohno, E. Stephen Buescher, Robert Roberts, Julia A. Metcalf, and John I. Gallin

Chronic granulomatous disease (CGD) is a genetically heterogeneous syndrome characterized by a microbial killing defect of polymorphonuclear leukocytes (PMNs) due to lack of superoxide O_2^- generation. Recent studies indicate that the neutrophil O_2^--generating system consists of at least two components, flavoprotein—flavin adenine dinucleotide (FAD)— and cytochrome b. We evaluate the cytochrome b and FAD content in PMN from 30 CGD patients. The method for quantitating cytochrome b was modified by using PMN sonicates incubated with azide plus hydrogen peroxide. With this approach, several absorption peaks corresponding to myeloperoxidase and eosinophil peroxidase, which overlap with peaks of cytochrome b, were obliterated from reduced-minus-oxidized spectra, whereas the peaks of cytochrome b were not and could be readily quantitated. Cytochrome b was detected in PMNs from all 24 normal adults (47.4 ± 2.9 pmol/7.5 × 10^6 cells), was absent in PMNs from 11 male CGD patients and one female CGD patient but was present in normal amounts in PMNs from nine male and nine female CGD patients. Stimulated nitroblue tetrazolium (NBT) tests performed on PMNs from mothers of CGD patients indicated that cytochrome b deficiency was associated with X-linked inheritance, except in one case in which probable autosomal recessive inheritance was demonstrated. The PMN NBT test of the mother of another male patient without cytochrome b deficiency suggested an X-linked form of inheritance. In related studies, the FAD content in PMN particulate fractions was reduced in 4 of 28 CGD patients studied. All four CGD patients with reduced FAD lacked cytochrome b. However, three patients with cytochrome b deficiency had normal FAD. Thus, the results indicate that PMN cytochrome b deficiency is observed in most X-linked and in some autosomal recessive CGD, that cytochrome b deficiency may be associated with FAD deficiency, and that cytochrome b and FAD are normal in most patients with non-X-linked CGD.

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POLYMORPHONUCLEAR leukocytes (PMNs) release superoxide anion (O_2^-), hydrogen peroxide, and other oxygen radicals when stimulated with particulate stimuli such as bacteria or fungi or with soluble stimuli such as phorbol esters, chemotactic peptides, and calcium ionophore.[1] Superoxide anion generated in the plasma membrane or phagosomal membrane by reduction of oxygen by electrons originating from neutrophil glucose-6-phosphate dehydrogenase (NADPH), dismutates to form hydrogen peroxide.[2,3] The hydrogen peroxide contributes to the microbicidal activity of neutrophils in cooperation with myeloperoxidase and halide.[4] Neutrophils from patients with chronic granulomatous disease (CGD) cannot produce superoxide and hydrogen peroxide when exposed to metabolic stimuli. As a result, patients with CGD are highly susceptible to bacterial infections. The disease may be classified into several hereditary types, including X-linked,[5] autosomal recessive,[5] and, possibly, autosomal dominant types.[6] Deficiency of NADPH,[7] glutathione peroxidase,[8] and NADPH oxidase have been thought to be the basis for the disease in different patients.

Recently, the NADPH oxidase has been considered a multicomponent system. A nonmitochondrial cytochrome b probably is involved. Some evidence indicates that the cytochrome b functions as a terminal component of the NADPH oxidase in donating electrons to oxygen.[9,10] The cytochrome b has dual subcellular localization and has been localized to the plasma membrane and an intracellular pool that cosediments with specific granules.[11–13] Flavoprotein, flavin adenine dinucleotide (FAD), is probably another component in the O_2^- generating system[14–18] and seems to be a proximal constituent of the electron-transport chain which accepts electrons directly from NADPH.[18]

Segal et al studied the content of cytochrome b in PMNs from 27 patients with CGD[19] and suggested that neutrophil cytochrome b deficiency was related to an X-linked inheritance of the disease. In addition, several reports indicate that the FAD content in the particulate fractions of neutrophils is decreased in some CGD patients.[16,17,20,21] Of eight patients reported previously with PMN FAD deficiency, seven patients had PMN cytochrome b deficiency. In the current study, the cytochrome b content in neutrophils from 30 CGD patients was measured by a modified method that removes an artifact of eosinophil peroxidase. In addition, FAD content was quantitated in 28 CGD patients. Although most patients with CGD who had cytochrome b deficiency had an X-linked pattern in their disease, in one family the inheritance was best explained as autosomal recessive. In another family with X-linked inheritance, neutrophil cytochrome b was normal. Of 28 patients examined, FAD was reduced in four patients whose neutrophils also lacked cytochrome b.

MATERIALS AND METHODS

Patients The diagnosis of CGD was determined by defective reduction of phorbol myristate acetate (PMA)-stimulated nitroblue tetrazolium (NBT) dye and impaired killing of *Staphylococcus aureus* by neutrophils from the patients.[6] Severe G6PD deficiency was excluded by demonstrating that the patients' cells responded to methylene blue with enhanced hexose monophosphate shunt activity.[6] Thirty patients from patients referred to the National Institutes of Health for evaluation of compromised host defenses were diagnosed as having CGD

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Preparation of cell sonicates. PMNs were collected by centrifugation on Ficoll-Hypaque and dextran sedimentation as previously described.[22] For one study, eosinophils were collected from the blood of a patient with microfilaria infection, as described elsewhere.[23] This method yields almost pure populations of eosinophils (>98% purity) Cells were suspended at a concentration of 7.5×10^6/mL in 50 mmol/L of 3-[*N*-morpholino] propane-sulfonic acid (MOPS), 100 mmol/L of KCl, pH. 7.0, sonicated for two cycles of ten seconds at 18 W at 4 °C, and solubilized by addition of sodium deoxycholate (0.1% of the final concentration).

Treatment of cell sonicates with azide plus hydrogen peroxide. Cell sonicates (7.5×10^6/mL) were incubated with 0.5 mmol/L of hydrogen peroxide plus 5 mmol/L of sodium azide at room temperature for 15 minutes. In some experiments, cell sonicates were incubated with 10 mmol/L of glucose plus 50 mU/mL of glucose oxidase in the presence of 5 mmol/L of azide at 37 °C for 30 minutes. The glucose–glucose oxidase system is an effective hydrogen peroxide generating system.[24] After incubation, the sonicates were cooled and kept in melting ice until spectral assay.

Reduced-minus-oxidized difference spectra. One milliliter of a sonicated sample (7.5×10^6 cells) was put in a cuvette. The absolute spectra of the sample were recorded from 600 nm to 400 nm with a Perkin-Elmer lambda-3 spectrophotometer and stored in an attached computer (Perkin-Elmer 3600 data station). The sample was reduced with the addition of solid sodium dithionite (1 to 2 mg). The absolute spectra of the reduced sample was recorded from 600 nm to 400 nm and stored. Reduced-minus-oxidized difference spectra were created by subtracting the absolute spectra of the sample from the absolute spectra of the reduced sample with the computer. Cytochrome b was measured in PMN sonicates treated with azide plus hydrogen peroxide as described in the first part of the Results section, using an absorption coefficient of 106 mmol/L^{-1} cm^{-1} at the Soret peaks[25] and expressed as pmol/7.5 $\times 10^6$ cells.

Subcellular fractionation. Subcellular fractions were collected from normal human neutrophils and from CGD neutrophils lacking cytochrome b by whole cell assays. The method for obtaining subcellular fractions with Percoll was described previously.[13] In brief, PMNs (1.5×10^8) were disrupted by nitrogen cavitation. The postnuclear cavitate was layered on a discontinuous gradient of 37.4% and 97.4% Percoll. Gradients were centrifuged for 15 minutes at 33,000 g. The postnuclear cavitate was separated into plasma membrane-rich, specific granule-rich, azurophil granule-rich, and cytosolic fractions on the basis of well-described enzyme marker assays described below. Collected fractions were separated from the Percoll and resuspended in a MOPS-KCl buffer in a concentration of 0.2 mg/mL of protein. Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate as a substrate.[22] Vitamin B_{12} binding protein was measured with ^{57}Co-vitamin B_{12} (Amersham, Arlington Heights, Ill).[22] Myeloperoxidase and cytochrome b in subcellular fractions were assayed with reduced-minus-oxidized difference spectra. An absorption coefficient of 75 mmol/L^{-1} cm^{-1} at 472 nm was used to quantitate myeloperoxidase.[26] Cytochrome b was measured with the samples treated with azide plus hydrogen peroxide, using an absorption coefficient of 106 mmol/L^{-1} cm^{-1} at the Soret peak.[25] The protein was determined with Coomassie blue as described previously.[27]

Assay of flavins FAD and flavin mononucleotide (FMN) were determined in the particulate fraction collected from activated PMNs as described previously.[20] PMNs (10^8 cells) were activated by incubation with 100 ng/mL of PMA at 37 °C for five minutes in Krebs-Ringer's phosphate buffer with 5.5 mmol/L of glucose, pH 7.4 After activation, cells were washed twice, suspended in 0.34 mol/L of sucrose, and sonicated for three cycles of ten seconds at 18 W at 4 °C. The sonicates were centrifuged at 500 g for ten minutes. The postnuclear supernatant was centrifuged at 27,000 g for 20 minutes at 4 °C. The pellet was resuspended in 0.34 mol/L of sucrose and recentrifuged at 27,000 g at 4 °C. The particulate fraction was washed once more and resuspended in 0.1 mol/L of potassium phosphate buffer with 0.1 mmol/L of EDTA, pH 7.7, to the concentration of 0.4 mg/mL of protein. FAD and FMN were quantitated by the method of Faeder and Siegel[28] Triton X-100 (Rohm and Haas, Philadelphia) was added to particulate fractions to a final concentration of 0.1%. The particulate fractions were immersed in boiling water for four minutes and cooled rapidly in melting ice water. Insoluble materials were removed by centrifugation in an Eppendorf microfuge for ten minutes at 4 °C. The fluorescence of the samples was measured with the excitation wavelength of 450 nm and the emission wavelength of 535 nm, using a Perkin-Elmer LS-5 fluorescence spectrophotometer. Thereafter, the pH of the solutions was lowered to 2.6 with 1 *N* HCl, and the fluorescence was then determined. Standard curves for flavins at pH 7.7 and pH 2.6 were determined with commercially obtained FAD and FMN (Sigma Chemical Co, St Louis).

Neutrophil functions. The PMA-stimulated NBT test[29] and superoxide production,[22] were performed as described previously using 20 ng/mL of PMA or 1 *u*mol/L of formyl-methionyl-leucyl-phenylalanine (FMLP) as the stimulus.

RESULTS

Modification of difference spectra assay used for measurement of cytochrome b. Several peaks were observed in reduced-minus-oxidized difference spectra from normal neutrophils (Fig 1). A prominent positive peak of absorption at 472 nm and a negative peak at 420 nm were due to absorption by myeloperoxidase.[30] A shoulder observed at 452 nm corresponded to the absorption of eosinophil peroxidase. A small positive peak at 558 nm and a prominent peak at 424 nm were the α and Soret peaks of cytochrome b, respectively.[10] Previous workers have used the α-peak (558 nm) to quantitate cytochrome b,[19] but the assay is difficult when PMNs contain many eosinophils, as occurs in leukocytes obtained from many patients with CGD since eosinophil peroxidase absorbs at 450 nm and 560 nm. For example, the difference spectra of eosinophils separated from a patient with hypereosinophilia (Fig 2) revealed a prominent positive

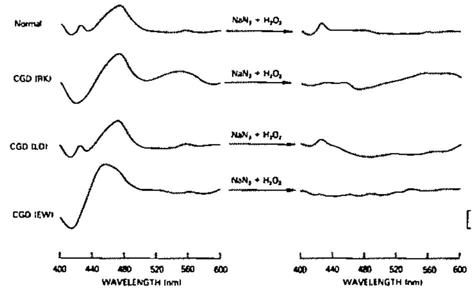

Fig 1. Reduced-minus-oxidized difference spectra of sonicates from normal and chronic granulomatous disease polymorphonuclear leukocytes (CGD PMNs). Normal or CGD PMNs (7.5×10^6/mL) in 3-[*N*-morpholino] propane-sulfonic acid (MOPS)-KCl buffer, pH 7.0 were sonicated at 18 W for two cycles of ten seconds. The sonicates were incubated with 5 mmol/L of azide and 10 mmol/L of glucose plus glucose oxidase (50 mU/mL) for 30 minutes at 37 °C. Tracings (left) show dithionite reduced-minus-oxidized difference spectra of a PMN sonicate; tracings (right column) show difference spectra of a PMN sonicate incubated with azide plus hydrogen peroxide generating system.

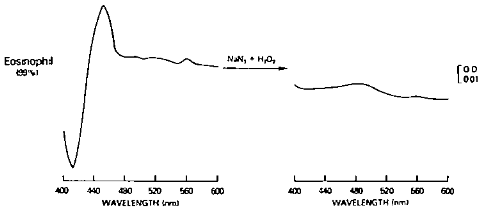

Fig 2. Reduced-minus-oxidized difference specta of eosinophil sonicates. The difference spectra of eosinophils was recorded in the same scale and the same number of cells as the polymorphonuclear leukocytes (PMN) sonicates shown in Fig 1. Tracing (left) shows dithionite reduced-minus-oxidized difference spectra of eosinophil sonicates; tracing (right) shows difference spectra of an eosinophil sonicate incubated with 5 mmol/L of azide plus 0.5 mmol/L of H_2O_2.

peak at 450 nm, a negative peak at 414 nm, and a small positive peak at 560 nm; these correspond to the maxima of the spectra of eosinophil peroxidase.[31] Difference spectra of PMN sonicates from one CGD patient (E.W.) showed a prominent shoulder at 452 nm due to a high content of eosinophil peroxidase (Fig 1). A small peak observed at 560-nm absorption, corresponding to eosinophil peroxidase, makes assay of cytochrome b at 558 nm impossible.

Therefore, to improve quantitation of cytochrome b, we developed a new technique to delete peaks of myeloperoxidase and eosinophil peroxidase. As shown in Fig 1 and Fig 2, the peaks of myeloperoxidase and eosinophil peroxidase were completely obliterated by treating the cell sonicates with azide plus hydrogen peroxide, and an α peak and a Soret peak of cytochrome b were clearly observed. Difference spectra of PMN sonicates from two male CGD patients suspected of X-linked CGD (R.K. and E.W.) are shown in Fig 1. Treatment of the sonicated sample with azide plus hydrogen peroxide obliterated the absorption peaks of myeloperoxidase and eosinophil peroxidase, clearly delineating the absence of the α and Soret peaks of cytochrome b. In a female patient with non-X-linked CGD (L.O.), absorption peaks for myeloperoxidase and cytochrome b were observed before treatment with azide plus hydrogen peroxide (Fig 1). After treatment with azide plus hydrogen peroxide, the α and Soret peaks of cytochrome b were seen.

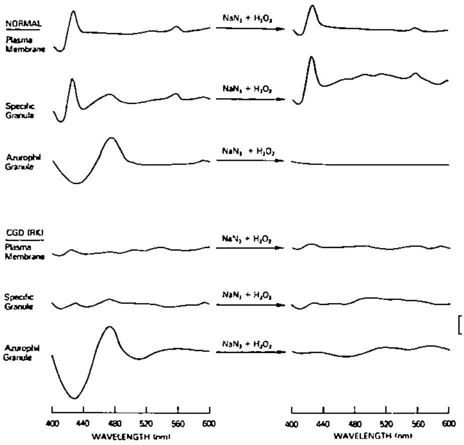

Fig 3. Reduced-minus-oxidized difference spectra of subcellular fractions isolated from normal and chronic granulomatous disease polymorphonuclear leukocytes (CGD PMNs). Normal PMNs and PMNs from a patient with CGD (R.K.) were disrupted by nitrogen cavitation. The subcellular fractions were separated from the postnuclear cavitates with Percoll-density gradients. The fractions were adjusted to 0.2 mg/mL of protein. The reduced-minus-oxidized difference spectra of subcellular fractions and those fractions treated with azide plus hydrogen peroxide were recorded.

To determine whether treatment with hydrogen peroxide plus azide affects the absorption of cytochrome b, we studied the effect of azide plus hydrogen peroxide on PMN subcellular fractions. Marker proteins in each fraction are shown in Table 1. The membrane-rich fraction was enriched in alkaline phosphatase. The specific granule-rich fraction contained a large amount of vitamin B_{12} binding protein. Myeloperoxidase was mainly enriched in the azurophil granule-rich fraction, but the specific granule-rich fraction also included a small amount of myeloperoxidase. Cytochrome b was located on both the plasma membrane-rich and specific granule-rich fractions as reported previously.[12,13] In Fig 3, the difference spectra of each fraction before and after treatment with azide plus hydrogen peroxide are shown. Following treatment with azide plus hydrogen peroxide, both the positive peak at 472 nm and the negative peak at 435 nm were eliminated, indicating that myeloperoxidase was successfully deleted from the azurophil granule-rich and specific granule-rich fractions. The height of a Soret peak (424 nm) of cytochrome b observed in the membrane-rich fraction, which lacked myeloperoxidase, was not decreased by the treatment. Therefore, this peak was used for all measure-

Table 1. Marker Proteins in Subcellular Fractions From Neutrophils

Fraction	Alkaline Phosphatase (mU/mg of Protein)	Vitamin B_{12} Binding Protein (ng Vit B_{12} Bound/mg of Protein)	Myeloperoxidase (nmol/mg of Protein)	Cytochrome b (nmol/mg of protein)
Normal				
Membrane	149 ± 32	0.80 ± 0.24	0.02 ± 0.01	1.38 ± 0.04
Specific granule	21 ± 3	3.83 ± 1.04	0.40 ± 0.02	0.73 ± 0.08
Azurophil granule	24 ± 4	0.47 ± 0.16	1.47 ± 0.15	0.01 ± 0.01
Cytosol	8 ± 1	0.21 ± 0.08	0 ± 0	0 ± 0
CGD (R.K.)				
Membrane	186	0.73	0	Trace
Specific granule	36	3.47	0.28	Trace
Azurophil granule	40	0.38	1.07	0
Cytosol	7	0.42	0	0

Separation of subcellular fractions and the assay of various proteins are described in Materials and Methods section. Data for the normal PMNs are mean ± SEM of nine preparations whereas CGD data are from a typical experiment.

ments of cytochrome b unless indicated otherwise. We did not select the α peak for measurement of cytochrome b because the height of the Soret peak was five times higher than the α peak.

Cytochrome b in subcellular fractions of CGD PMNs. With the above method, the difference spectra of subcellular fractions from CGD cells that lacked cytochrome b were examined. Distribution of alkaline phosphatase, vitamin B_{12} binding protein, and myeloperoxidase in each subcellular fraction was the same as in normal PMNs (Table 1). Although peaks of myeloperoxidase absorption were clearly observed, the absorption peaks corresponding to cytochrome b were not seen (Fig 3). Only a minimal positive peak at 429 nm and its negative peak at 410 nm were observed in the plasma membrane-rich and specific granule-rich fractions after azide plus H_2O_2 treatment (Fig 3). These small peaks at the Soret region probably come from contaminated hemoglobin or from hemochromes in the mitochondria or microsome fractions. The contribution of substances other than cytochrome b to the Soret peak observed in the difference spectra of the subcellular fractions was negligible.

Measurement of cytochrome b in normal and CGD PMNs. PMNs from normal adults contained 47.4 ± 2.9 (mean ± SEM, n = 24) pmol of cytochrome b per 7.5 × 10^6

Table 2. Cytochrome b and FAD in PMN From Patients With CGD

Case	Cytochrome b (pmol/7.5 × 10^6 Cells)	FAD (pmol/mg of Protein)	NBT in Mother's PMNs (% Positive)
Male CGD without cytochrome b			
M.J.	0*	24*	21
D.H	0*	90	45
E W.	0*	85	21
R W.	0*	34*	61
S.S	0*	128	60
P K †	0*	68	70 Mother, 47 (maternal grandmother)
R K †	0*	55	Same as above
D H	0*	120	50
E H.‡	0*	24*	67
S.H.‡	0*	25*	Same as above
T.H.	0*	68	NT
Male CGD with cytochrome b			
H.P.	40.1	122	100
T A	51.9	144	100
C.T.	35.4	73	NT
R.G.	23.0	145	98.0
B.J.§	32 4	153	NT (deceased)
M B.¶	40.1	54	NT (deceased)
J H	20 0	48	100
P.R	55.0	135	99
R D	74.6	59	56, 82
	41.4 ± 5 7‖		
Female CGD with cytochrome b			
A A	31 3	NT	NT
L.O.	32.4	NT	100
D J.§	45 4	143	NT (deceased)
S.J.§	35.4	155	NT (deceased)
K.L ¶	70 7	113	NT (deceased)
J.M.**	53 1	143	100
M.M.**	46 0	143	Same as above
L.H ††	73.7		100
C.H.††	49 7	105	100
	48.6 ± 5 1‖		
Female CGD without cytochrome b			
A.V	0*	101	98

Cytochrome b in PMNs from CGD patients was measured from difference spectra of PMN sonicate treated with azide plus hydrogen peroxide as shown in Fig 1.

*$P < 0\ 05$ *v* normal, Student's *t* test.

‖No significant difference as compared to normal

†Brothers; ‡brothers; §siblings (D J and S J . nonhomozygous twins), ¶siblings; **homozygous twins, ††C.H is a fraternal aunt of L H , suspected of autosomal dominant inheritance.[8] Parents of the patients (H.P., M B., K L., and C.T.) are first cousins. Cytochrome b content in PMNs from 24 normal adults was 47.4 ± 2.9 pmol/7 5 × 10^6 cells. FAD content of PMN particulate fraction from 14 normal adults was 104.7 ± 9.2.

NT, not tested.

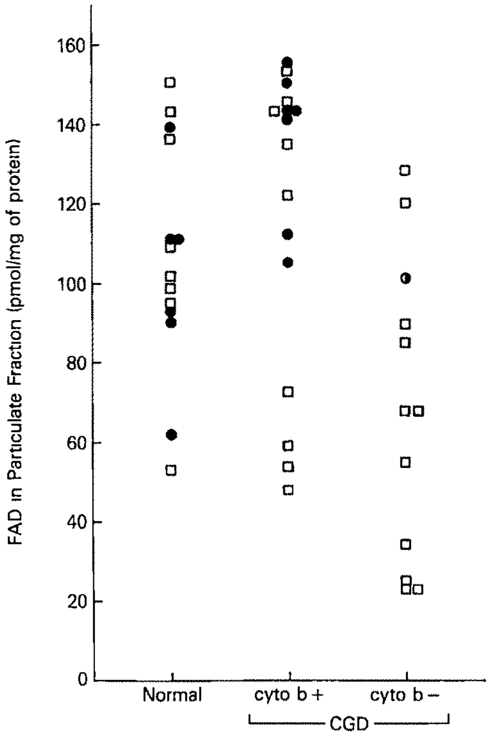

Fig 4. Flavin adenine dinucleotide (FAD) in the particulate fraction of normal and chronic granulomatous disease (CGD) neutrophils. Polymorphonuclear leukocytes (PMNs) were activated by incubation with 100 ng/mL of phorbol myristate acetate (PMA) for five minutes at 37 °C. The cells were washed twice and sonicated. The postnuclear supernatant was centrifuged at 27,000 g for 20 minutes at 4 °C. The pellet, (PMN particulate fraction) was assayed for FAD as described in the Materials and Methods section. Females (●); males (□); patient A.V. (O).

cells. Cytochrome b was absent from PMNs from 11 male patients with CGD (Table 2). Cytochrome b was detected in normal amounts in PMNs from nine other male patients with CGD (41.4 ± 5.7 pmol/7.5 × 10^6 cells) (Table 2). PMNs from nine female patients with CGD also contained normal amounts of cytochrome b (48.6 ± 5.1 pmol/7.5 × 10^6 cells), although PMNs from a female patient (A.V.) totally lacked cytochrome b (Table 2).

FAD in neutrophil fractions. FAD in particulate fractions of normal PMNs was 104.7 ± 9.2 pmol/mg of protein (n = 14). However, significantly reduced FAD was observed in four CGD patients with associated cytochrome b deficiency (Fig 4) The other patients had normal PMN FAD. The data pertaining to FAD in each CGD patient are included in Table 2.

A subcellular deficiency of FAD has been suggested as the basis of a defect in some patients.[21] To assess this possibility, neutrophils from three patients with low normal FAD, (M.B., J.H., and R.D., Table 2), were fractionated into cytosol-, membrane-, specific granule-, and azurophil granule-enriched fractions as described in Table 1. All fractions from the CGD patients contained FAD; the only possible deficiency noted was in the specific granule-enriched fraction of M.B., which contained 101 pmol/mg protein *v* 219 ± 44 pg/mg protein for the three normal subjects. Thus, although a subcellular deficiency of FAD may exist in one of these patients, the magnitude of the defect is not dramatic.

A family with probable autosomal recessive cytochrome b deficiency (Table 3). Although most of our patients with cytochrome b deficiency had an X-linked recessive pattern of inheritance, the disease appeared to be inherited in an autosomal recessive pattern in one female patient (A.V.). The PMA-stimulated NBT test confirmed that the patient PMNs could not reduce NBT after stimulation. The NBT scores of PMNs from the mother and father and three male siblings were normal. Superoxide generation was totally absent in the patient's PMNs. PMNs from the mother, father, and two siblings showed a common pattern of superoxide production; normal response to PMA but reduced response to fMet-Leu-Phe.

The cytochrome b of the patient's PMNs was not detected in three separate examinations. The cytochrome b content of PMNs from the mother and the father were significantly reduced to about half-normal (Table 3) although PMNs from two of the patient's siblings with somewhat reduced O_2^- production to fMet-Leu-Phe contained normal amounts of cytochrome b. FAD of PMN particulate fractions were normal in every member of the family, including the patient.

DISCUSSION

The most commonly used method for quantitating cytochrome b is the method of Segal et al who used the α peak of cytochrome b (558 nm) absorbance to measure neutrophil

Table 3. Study of a Family of a Female CGD Patient With Cytochrome b Deficiency

	(%) Positive NBT†	O_2^- Production*			
		FMLP (1 µmol/L)	PMA (20 ng/mL)	Cytochrome b (pmol/7 5 × 10^6 cells)	FAD (pmol/mg Protein)
Normal	>90.0	3.56 ± 0.70 (8)	8.75 ± 1 16 (8)	45 8 ± 2.9 (22)	105 ± 9 (14)
CGD (A.V.)	0	0 ± 0 (3)‡	0 ± 0 (3)§	0 ± 0 (3)§	101
Mother	98 0	1 46 ± 0.57 (3)	8.00 ± 3.81 (3)	18 2 ± 2 4 (3)¶	34
Father	98 0	1 44 ± 0.13 (3)	7.66 ± 2.06 (3)	15 8 ± 0 7 (3)¶	106
Brother	99.0	1.05 ± 0.54 (3)	8.94 ± 4.79 (3)	30.7	56
Brother	97.5	1.68 ± 0.30 (3)	8 94 ± 2.44 (3)	31.4	67
Brother	99 0	4.61 ± 0.91 (2)	7 91 ± 0.67 (2)	31.4	111

Data show the mean ± SEM of the number of experiments in parentheses.

*Maximal rate nmol/10^6 cells/1 min.

†NBT reduction was examined on PMN stimulated with PMA (20 ng/mL).

$P < 0.01$

‡$P < 0$ 02, §$P < 0.001$; ¶$P < 0$ 01. *v* normal

cytochrome b.[19] With this approach, a peak of eosinophil peroxidase (560 nm) overlaps the α peak of cytochrome b,[25] making it difficult to measure cytochrome b in PMNs containing eosinophils, which in our experience are frequently mildly elevated in CGD. We overcame this problem by treating PMN sonicates with azide and hydrogen peroxide. This method obliterated the absorption peaks of myeloperoxidase and eosinophil peroxidase without significant alteration of cytochrome b absorption. We have shown previously that azide plus hydrogen peroxide irreversibly inactivates the activity of myeloperoxidase and eosinophil peroxidase.[32]

PMN cytochrome b of 24 normal adults and 30 CGD patients was measured. Cytochrome b was detected in cells from each normal adult (47.4 ± 2.9 pmol/7 5 × 10^6 cells, n = 24). This value was slightly lower than those described by Segal et al,[19] probably because the absorption at 558 nm they measured represented the α peak of cytochrome b plus the 560 mm absorption peak of eosinophil peroxidase. In 11 of 20 male CGD patients, PMN sonicates lacked cytochrome b (Table 2). Neutrophils from mothers of the patient group were examined for the stimulated NBT test. A mosaic pattern of normal and abnormal neutrophils was found in all patients, indicating heterozygous carriers and an X-linked trait of the disease (Table 2). On the other hand, cytochrome b was present in neutrophils from nine other males with CGD. Neutrophils from five mothers had a normal NBT score and one, R.D., had a mosaic pattern (Table 2). Cytochrome b content was normal in PMNs from nine female patients with CGD. The neutrophil NBT test from each mother in this group of female CGD patients was normal (Table 2).

As reported previously, X-linked transmission and autosomal recessive transmission of CGD are commonly observed,[5,6] and one family with autosomal dominant inheritance has been reported.[6] A recent study suggested that the autosomal recessive form of CGD has less severe symptoms than does the X-linked form of the disease.[33] We saw no difference in symptoms among our patient groups. However, this may be because the use of prophylactic bactrim therapy[6] makes comparison of disease severity among the patient groups difficult to interpret. Segal et al suggested that the cytochrome b content in neutrophils from CGD patients is valuable for determining the type of genetic transmission.[19] Our study supports Segal's conclusion except for two patients. One patient (A.V.) is a female with cytochrome b deficiency. Extreme lyonization of a heterozygote is not a likely explanation for the inheritance in A.V. because the NBT score of the mother's PMNs was not compatible with X-linked CGD. Furthermore, the cytochrome b content in PMNs of both her mother and father was reduced to half-normal, strongly suggesting an autosomal recessive type of cytochrome b deficiency. In addition, the superoxide production of the mother and father's PMNs was reduced when FMLP, but not PMA, was used as a stimulant (Table 3). Abnormal superoxide production was also observed in PMNs from two of her siblings, although PMN cytochrome b of the siblings was not sufficiently reduced to indicate that they were heterozygotes (Table 3). Another description of a family with an autosomal form of cytochrome b deficiency appeared in a recent paper.[34] Thus, a phenotype of neutrophil cytochrome b deficiency can be separated into two genetic types, X-linked or autosomal recessive. The synthesis of mature cytochrome b must require several processes; putative gene activator proteins, transcription, RNA processing, nuclear to cytoplasmic transport of mRNA, translation, and posttranslational modification including incorporation of the heme moiety. If any step is defective or lacking, cytochrome b will be undetectable by spectroscopy. The identification of families with autosomal recessive cytochrome b inheritance furthers our understanding of the genetic mechanism of neutrophil cytochrome b production Probably a structural gene for cytochrome b will be located on an autosomal chromosome. Thus, a mutation of one of the homologous autosomal chromosomes (heterozygote) would lead to a half-normal cytochrome b. The X chromosome may have a gene encoding a putative cytochrome b gene activator protein that may positively switch on the transcription of the cytochrome b gene.[35] Alternatively, the X chromosome may have an "enhancer element" that ensures accurate and efficient initiation of transcription.[36] Further understanding of the molecular defect of CGD patients with cytochrome b deficiency will require purification of cytochrome b and cloning of the cytochrome b gene.

The other patient who did not fit the generalization that all X-linked CGD patients have cytochrome b deficiency is a male CGD patient (R.D.) strongly suspected of X-linked CGD from the NBT score of his mother's PMNs; however, the patient's PMNs contained normal amounts of cytochrome b (Table 2). Moreover, the mother's PMNs also contained normal amounts of cytochrome b (49.7 pmol/ 7.5 × 10^6 cells). This is important because heterozygotes of X-linked cytochrome b deficiency should show reduced PMN cytochrome b according to the NBT score.[19] The patient's PMNs also contained normal FAD. Therefore, in patient R.D., deficiency of another substance controlled by the X-chromosome is suspected. In this respect, a recent report suggested a requirement of a cytosolic factor for O_2^- generation,[37] which may be missing in patient R.D.

Several publications reported reduced FAD in CGD neutrophils. Cross et al reported three cases of X-linked CGD, in whom neutrophil membrane fractions contained 50% of the normal level of FAD[16]; these patient's neutrophils also lacked cytochrome b. In contrast, in three cases with autosomal recessive CGD, FAD and cytochrome b were normal. Cross et al suggested that there is a close association between FAD and cytochrome b. Gabig and Lefker reported three male CGD patients with FAD deficiency of the neutrophil particulate fraction[20]; two of the patients also had neutrophil cytochrome b deficiency. Borregaard and Tauber reported two CGD patients whose neutrophils showed a deficiency of FAD and absent cytochrome b in neutrophil fractions rich in specific granules although these patients' PMNs had normal quantities of FAD in fractions containing plasma membrane and cytosol.[21] We determined the FAD content in the particulate fractions of PMNs from 28 patients with CGD. FAD was normal in 14 patients whose PMNs contained normal levels of cytochrome b (Fig 4). CGD patients with PMN cytochrome b deficiency were separated into two groups; significantly reduced FAD (~25% of normal) was observed in four patients, whereas FAD was normal in eight patients (Table 2, Fig 4). The results support a close relationship between cytochrome b deficiency and reduced FAD content but indicate that cytochrome b deficiency with normal FAD also exists.

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TCA: A Polymorphic Genetic Marker in Leukemias and Melanoma Cell Lines

By A van Leeuwen, P.I. Schrier, M.J. Giphart, I.A. Noordermeer, D.J. Ruiter, P. Rubinstein, and J.J. van Rood

TCA (T Cell system A) is a di-allelic system of HLA-like antigens encoded by genes located about 15 cM telomeric to HLA-A. In normal individuals, TCA antigens are only expressed on a subpopulation of T cells, the T_G lymphocytes. We now report on the expression of TCA on leukemias and other malignancies. An increased proportion of cells carrying the TCA phenotype was encountered in testing peripheral blood lymphocytes from patients with acute lymphoblastic T-cell leukemia (T-ALL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). In contrast, patients with B-cell malignancies such as chronic lymphatic leukemia (CLL) and hairy cell leukemia (HCL) or non-T/non-B common acute lymphoblastic leukemia (common ALL) had normal proportions of TCA-positive lymphocytes. Quantitatively different levels of TCA expression are found on some melanoma cell lines and others are TCA negative. These variations are independent of the expression of HLA Class I antigens by the same cells. The expression of TCA antigens by malignant nonlymphoid cells suggests that this system may code for differentiation markers, important in the biology of neoplastic transformation.

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WE HAVE recently described a diallelic system of antigens coded by genes that map 15 cM telomeric to HLA-A in chromosome 6.[1,2] These antigens, designated TCA-1 and TCA-2, are comprised of an α-chain (MW $\simeq$ 42.000) and β_2-microglobulin, and are, therefore, homologous of the murine H-2–linked Tla-Qa antigens. The expression of Tla and Qa in malignant cells has been reported to be different from that in normal cells,[3] which suggests the participation of this genetic system in the biology of neoplasia. The identification of TCA antigens by serological methods is complicated by the frequent coexistence of HLA antibodies in the same sera.

We have, therefore, developed an approach by which antibodies against TCA or other possible equivalents of Qa or Tla could be identified.[1,4] Sera were collected from highly immunized individuals (mainly multiparous women) who carried the very frequent HLA-A1, B8, DR3 haplotype. The sera were then tested against a panel of HLA-A1, B8, DR3–homozygous cells that guaranteed that any antibodies reactive with Class I or Class II antigens would not find a target. In this manner, sera containing interesting antibodies were identified and the unwanted antibodies subsequently removed by absorption to permit their use as conventional typing reagents. Such reagents have allowed us to describe four non-HLA antigens belonging to two distinct immunogenetic systems.[2] One, the TCA system, is normally expressed only by the T_G subpopulation of peripheral blood lymphocytes. Since those cells comprise only 10% of peripheral blood lymphocytes, an enrichment procedure had to be used as well.

The discovery of TCA allows us to approach the study of the chromosomal region distal to HLA-A in humans. Data from the murine work suggests that this region may contain additional Class-I-like (or class IV[5]) genes with interesting functional properties.

We have, therefore, investigated the expression of TCA antigens by neoplastic cells. Our results reveal that the proportion of TCA-positive cells is consistently increased in patients with either acute lymphoblastic T cell leukemia (T-ALL) or myeloid malignancies such as acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). TCA, however, is not increased in patients with B cell malignancies, such as chronic lymphatic leukemia (CLL) and hairy cell leukemia (HCL) or non-T/ non-B common acute lymphoblastic leukemia (common ALL). We also found TCA to be expressed by some but not all melanoma cell lines in which it occurs independently of the expression of conventional HLA antigens. One of us[6] has previously described that loss of expression of those antigens by melanoma tumors may be associated with a poor prognosis.

MATERIALS AND METHODS

Antisera Sera, selected as described elsewhere,[1] were absorbed with EBV-transformed B cell lines derived from the respective husbands of the serum donors. This procedure, which eliminates HLA-A, -B, -C, and DR antibodies, had also been described previously.[1]

Normal lymphocytes. T lymphocytes were separated from Ficoll-Isopaque–prepared mononuclear cells by forming sheep red cell rosettes. T_G–enriched suspensions were then isolated using theophylline[4] which dissociates the rosettes formed by non-T_G cells.

Leukemic lymphocytes. Fresh as well as frozen peripheral blood lymphocytes from 62 leukemia patients were tested Ten cases were diagnosed as CLL, five as HCL, nine as common ALL, 16 as AML, nine as CML, two as monocytic leukemia (Mon L), and 11 as T-ALL The Ficoll-Isopaque separated suspension was not fractionated further

Complement-dependent cytotoxicity (CDC) technique. The CDC test was described elsewhere [7] In short, cells were incubated for one hour with one volume of antiserum at 10 °C followed by two hours incubation with five volumes of complement at 20 °C. Ethidium bromide was used as an indicator. Each reaction was scored according to the percentage of cells killed. A negative control was used to define negative and positive reactions. It consists of testing the target cells with platelet-absorbed AB serum plus complement· a positive reaction results in at least twice the percentage of dead cells observed in the AB control. Cytotoxicity scores were calculated

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according to the proportion of dead cells and are presented here together with an indication of their variability in the replicate determinations Between 200 and 500 lymphocytes were evaluated for viability in each test

Melanoma cell lines Melanoma cell lines were grown in DMEM supplemented with 10% fetal calf serum and trypsinized from the plastic dishes just before assaying for cytotoxicity with anti-HLA and anti-TCA sera. Mel 2a is a cell line established from an axillary lymph node metastasis from a 43-year-old female patient, passage 400. O-Mell II is a cell line from cells, isolated from pleural exudate of a 45-year-old male patient, passage 40. This patient was treated with DTIC. Mel 57 is a cell line derived from a local skin metastasis from the leg of a female patient that died in 1975 with a superficial spreading melanoma [8] Passage 120. Cell line IGR is established from an inguinal lymph node metastasis from a 30-year-old male patient. The original tumor was amelanotic of epithelial origin [9] Passage 50. CMel 136 is a cell line derived from the metastasis of the axillary lymph node from a 35-year-old male patient who had a primary melanoma of the nipple which had been excised two months earlier. CXCMel is a cultured xenograft of CMel 136 in Balb C nude mice

RESULTS

Leukemia patients. Peripheral blood lymphocytes and blasts from leukemia patients were tested with anti-TCA sera. The expression of TCA on leukemic cells were measured by the complement-dependent cytotoxicity (CDC) reaction of alloantisera and peripheral blood lymphocytes after Ficoll-Isopaque separation. A partial list of results is shown in Table 1. Patients with B cell malignancies (CLL and HCL) and non-T/non-B Common ALL gave similar patterns of cytotoxicity to those observed with unseparated lymphocytes from healthy individuals. In contrast, nonenriched lymphocytes from the 11 patients with T-ALL (three are shown in Table 1) and the two with monocytic leukemia tested to date show a highly increased proportion of TCA positive cells (2 to 8 times the normal level, see Table 1). In the majority of cases of AML and CML (AML 1°, AML 2, AML 3, CML 2, CML 3), a highly increased expression of TCA was also found (Table 1).

The levels of TCA reactivity of AML 1⁺ and CML 1 in nonleukemic phase were similar to the control, but blood collected from one of these patients in a subsequent leukemic phase (AML 1°, Table 1) demonstrated TCA antigens in a high proportion of the mononuclear cells. Two additional cases of reduced expression of TCA in remission of leukemia and their reappearance during leukemic phases have been encountered. We postulate, therefore, that enhanced expression of TCA is associated exclusively with the leukemic phase in patients with T-ALL, Mon L, AML, and CML and not with B cell or common AL leukemias.

It is evident that the levels of reactivity with the negative control serum (AB*) are variable, ranging from 4% to 21%. The use of frozen cells or the increased sensitivity to complement of leukemic cells from those patients may explain these higher negative control values. Especially in the monocytic leukemias and in the T-ALL patients the control values (AB*) are high; however, a clear increase in the percentage

Table 1 Percent Reactivity of Anti-TCA Sera With Peripheral Blood Lymphocytes From Leukemia Patients and Normal Controls

	Anti-TCA1 Sera				Anti-TCA2 Sera				TCA Phenotype
	29	24	15	22	14	23	32	AB•	
Controls N = 25									
Unseparated Cells	4 ± 1	3 ± 1	3 ± 1	4 ± 2	4 ± 2	3 ± 2	3 ± 1	4 ± 1	X
T_G enriched	25 ± 3	23 ± 2	20 ± 1	18 ± 2	29 ± 2	15 ± 2	20 ± 1	6 ± 1	1, 2
Patients[x]									
Diagnosis									
CLL 1	4 ± 1	8 ± 1	3 ± 1	4 ± 1	4 ± 1	ND	4 ± 1	4 ± 1	X
CLL 2	8 ± 1	8 ± 3	6 ± 1	6 ± 1	5 ± 1	ND	8 ± 1	5 ± 2	X
HCL	3 ± 1	4 ± 1	3 ± 1	3 ± 1	3 ± 1	ND	3 ± 1	5 ± 2	X
HCL	10 ± 3	7 ± 1	9 ± 2	8 ± 1	8 ± 1	10 ± 2	8 ± 2	8 ± 1	X
Common ALL 1	6 ± 1	7 ± 1	6 ± 1	7 ± 1	7 ± 1	6 ± 1	6 ± 1	6 ± 1	X
Common ALL 2	8 ± 2	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	X
AML 1⁺	8 ± 2	15 ± 1	13 ± 3	ND	15 ± 2	16 ± 2	13 ± 3	12 ± 2	X
AML 1°	30 ± 2	31 ± 1	39 ± 6	29 ± 4	40 ± 2	36 ± 1	32 ± 2	12 ± 1	1, 2
AML 2	53 ± 8	53 ± 3	53 ± 3	50 ± 5	ND	21 ± 1	20 ± 1	15 ± 3	1
AML 3	24 ± 5	28 ± 2	70 ± 8	ND	39 ± 6	57 ± 10	39 ± 7	8 ± 2	1, 2
CML 1	9 ± 1	12 ± 1	10 ± 1	11 ± 1	12 ± 1	10 ± 1	8 ± 2	8 ± 1	X
CML 2	18 ± 1	14 ± 1	18 ± 4	20 ± 2	40 ± 2	43 ± 7	37 ± 1	17 ± 1	2
CML 3	57 ± 4	60 ± 2	54 ± 3	49 ± 1	57 ± 3	69 ± 3	54 ± 4	9 ± 1	1, 2
Mon L	42 ± 3	55 ± 5	40 ± 1	38 ± 2	32 ± 2	35 ± 1	49 ± 1	11 ± 4	1, 2
Mon L	50 ± 1	55 ± 3	48 ± 1	55 ± 3	22 ± 1	ND	21 ± 1	21 ± 1	1
T-ALL	74 ± 5	83 ± 1	88 ± 2	84 ± 2	77 ± 5	77 ± 1	74 ± 3	17 ± 2	1, 2
T-ALL	24 ± 3	35 ± 1	38 ± 1	34 ± 1	32 ± 6	31 ± 6	34 ± 2	11 ± 2	1, 2
T-ALL	ND	50 ± 1	55 ± 1	52 ± 2	17 ± 1	23 ± 2	21 ± 3	17 ± 4	1

X TCA antigens are undetectable in the unseparated cells.

[x] unseparated cells.

⁺AML patient in nonleukemic phase

°same patient as AML 1⁺ but now in leukemic phase.

•platelet-absorbed serum used as negative control.

Table 2 Total Number of Leukemia Patients Tested for the Expression of TCA

Diagnosis	Increased Expression	Low Expression
CLL	0	10
Hairy CL	0	5
Common ALL	0	9
AML	11 (leuk phase)	5 (nonleuk phase)
CML	8 (leuk phase)	1 (nonleuk phase)
Mon L	2	0
T-ALL	11	0

Ficoll-Isopaque–isolated lymphocytes were defined as showing "increased expression" when a clear TCA pattern was found, and "low expression" when no TCA antigens were detected, using unseparated lymphocytes.

of positive cells could be observed. Because the reactions were read with an inverted microscope containing phase contrast, it could be demonstrated clearly that the positive reactions were selectively due to lysis of the blast cells in the peripheral blood lymphocyte suspension. Moreover, since the two-color fluorescence technique was used it was also obvious that B cells were not lysed.

The last column of Table 1 gives the TCA phenotypes of the patients with increased TCA antigenic expression. Patients with the three possible TCA phenotypes, ie, both homozygotes and the heterozygote classes, were encountered in the proportions expected from the Hardy-Weinberg equilibrium. Table 2 summarizes the TCA results with leukemic patients obtained to date. All of the leukemia patients showed normal HLA Class I and Class II antigen expression, as expected from previous experience.

Melanoma patients Since non-T cell leukemias, such as AML, also show TCA expression, we were interested in determining whether or not TCA is also expressed by malignancies other than those of hematopoietic origin. Consequently, we tested a number of melanoma cell lines. The expression of HLA in malignant melanoma has been reported to be highly variable[6] and Table 3 shows that four out of six melanoma cell lines do not react with anti-Bw4/Bw6 sera.

Table 3. Expression of HLA-B (Class I) and TCA on Melanoma Cell Lines as Demonstrated by the Reaction Patterns With Different Anti-Bw4, Anti-Bw6, Anti-TCA1, and Anti-TCA2 Sera

Melanoma Cell Lines	Anti–Class I		Anti-TCA	
	Bw4	Bw6	1	2
Mel 2a*	0	0	0	0
0-Mel II*	0	0	0	0
Mel 57	5	5	0	0
IGR	0	5	3	0
CMel 136*	0	0	4	4
CxCMel 136*	0	0	3	2

All melanoma cell lines were tested for HLA antigens using the standard NIH technique.[14] For TCA typing the CDC technique was used as described in Materials and Methods The scores used from 0 to 5 indicate: 0 = 0–10% dead cells, 1 = 11–20%, 2 = 21–30%, 3 = 31–50%, 4 = 51–80%, and 5 = 81–100%.

Bw4 and Bw6 are representative for the HLA-B locus All normal peripheral blood lymphocytes react at least with one of the two anti-Bw4 or anti-Bw6 sera.

*These cell lines are also unreactive with other HLA-A,-B, or-C antisera.

Those cell lines were also unreactive with other HLA-A, -B, or -C antisera These negative serological results were further confirmed by a highly reduced transcription of Class I MHC genes as detected on Northern blots (P.I. Schrier, unpublished observations). Of these HLA-A, -B, -C negative lines, CMel 136 and CXCMel 136 show high expression of TCA*1 and TCA*2. Two other cell lines (IGR and Mel 57) that have easily detectable Class I HLA-A, -B, -C (Table 3) differ in their TCA status. One line does not express TCA, while another is positive for TCA*1. The cell lines Mel 2a and O-Mel II neither express Class I nor TCA.

The reciprocal expression of TCA and HLA on CMel 136 and, for instance, Mel 57, stresses that Class I HLA-A, -B, -C, and TCA are clearly separate determinants on the membrane of the tumor cells. However, they are not mutually exclusive because cell line IGR, which has a high Class I expression, also expresses TCA.

DISCUSSION

The presence of TCA antigens in T cell leukemias might be expected from their normal T_G counterparts. It is, however, surprising that this system is expressed by all T cell leukemias studied, which suggest that either at least some normally TCA-negative cells may become positive as a result of neoplastic transformation or that the increased TCA expression is the result of a clonal expansion of TCA-positive cells in the peripheral blood. A similar finding with several myelocytic leukemias supports these suggestions. The association of TCA expression with neoplasia is not restricted to cells of the hematopoietic system, since at least some melanoma cell lines display high levels of TCA antigen expression. These cell lines, however, are rather heterogeneous with respect to the expression of HLA and/or TCA antigens. This is not a result of the well-known HLA variability in melanomas since TCA+, HLA (−); TCA(−), HLA+; TCA+, HLA+; and TCA (−), HLA(−) cell lines have been identified.

We can only speculate about the role of the increased expression of TCA on tumor cells. Malignant melanoma is a disease that elicits antibody to tumor-associated antigens and autologous cytotoxicity in some individuals[10] and might, therefore, be considered as a particularly immunoreactive human tumor. The low expression of Class I MHC molecules on the membrane may interfere with Class I–restricted recognition of tumor cells by cytotoxic T cells, thus allowing the tumor cells to evade the host's immune defense Examples of such a mechanism in experimental animal models have been described.[11–13] In this report we have shown that, in some instances, the decreased expression of Class I is associated with the appearance of new Class I–like determinants, such as TCA.

It remains to be demonstrated whether this also interferes with CTL recognition.

In this context, it is probably relevant that especially invasive melanoma metastases display low expression of HLA-A, -B, -C,[6] and accorded unusually low levels of lymphocytic infiltration in the patients. Accurate quantitation of the expression of TCA in these tissues is presently under investigation, to see whether TCA is also highly expressed in these tumors as has been found on the leukemic blasts. The results so far suggest that two mechanisms are involved in malignancies: (1) a decreased Class I expression interfering with lymphocytotoxicity, and (2) an increased TCA expression (along with any pattern of HLA expression).

A thorough characterization of TCA antigens and their regulation in tumor cells is, therefore, necessary and may provide a clue to their role in human malignancies.

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Effect of Activated Lymphocytes on the Regulation of Hematopoiesis: Enhancement and Suppression of In Vitro BFU-E Growth by T Cells Stimulated by Autologous Non-T Cells

By Mine Harada, Shinji Nakao, Kunio Kondo, Kazuaki Odaka, Mikio Ueda, Shintaro Shiobara, Kosei Matsue, Takao Mori, and Tamotsu Matsuda

Autologous mixed lymphocyte culture (AMLR) is an immunologic response with memory and specificity and plays a role in immune regulation. Effects of T cells activated by AMLR were studied in the regulation of in vitro erythropoiesis. AMLR-activated T cells were cocultured with autologous non-T, nonphagocytic peripheral blood mononuclear cells for assaying erythroid progenitor cells (BFU-E). T cells activated for 3 days in AMLR showed significant enhancement of in vitro colony growth by BFU-E. In contrast, activated T cells from day 7 AMLR caused significant suppression of BFU-E growth. Both enhancing and suppressing activities of AMLR-activated T cells were mediated by an Ia-positive and radiosensitive population within the OKT4$^+$ subset. These observations suggest that AMLR-activated T cells may play a role in the immune-mediated regulation of in vitro erythropoiesis. It is also suggested that heterogeneous T-cell subsets may exert regulatory functions in the regulation of in vitro hematopoiesis.

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T CELLS play an important role in the regulation of hematopoiesis.[1-5] Recently we have shown that T cells activated in vitro by concanavalin A or alloantigens in mixed lymphocyte reaction (MLR) could suppress in vitro colony formation by erythroid and/or myeloid progenitor cells.[6,7] Similar data have been reported by several investigators.[8-11] Concanavalin A and alloantigen stimulation is well known for its capacity to induce suppressor cells that operate in immune systems, such as in vitro immunoglobulin synthesis and MLR.[12-14] Another population of T cells that may affect the immune regulation is autoreactive T cells, which will proliferate in response to stimulation by autologous non-T cells in autologous MLR (AMLR).[15,16] AMRL is an immunologic response with memory and specificity[16,17] and T cells activated in AMLR have some immunoregulatory functions.[18] We investigated the effects of AMLR-activated T cells on immature erythroid progenitor cells (erythroid burst-forming unit, BFU-E) and found that these activated T cells exerted dual regulatory functions for in vitro colony growth by peripheral blood BFU-E.

MATERIALS AND METHODS

Preparation of T cells and non-T cells. Heparinized peripheral blood from normal volunteers was diluted with an equal volume of RPMI 1640 medium (GIBCO, Grand Island, NY), layered over a Ficoll-Hypaque density gradient, and centrifuged at 400 × G for 30 minutes. Peripheral blood mononuclear cells (PBMC) were obtained from the interface and washed twice with RPMI 1640. Then T cells and non-T cells were separated from PBMC by using the rosette sedimentation technique.[19] This procedure was repeated twice. T cells obtained in this way were >98% positive for T3 as determined by OKT3 monoclonal antibody (Ortho Pharmaceutical Corp, Raritan, NJ) staining on a FACS analyzer (Becton Dickinson, Mountain View, Calif) while non-T cells contained ≤5% OKT3-positive cells.

Isolation of T-cell subsets An OKT4- or OKT8-positive cell fraction was isolated by negative selection. T cells (1×10^7/mL) were mixed with 50 μg/mL of OKT4 or OKT8 monoclonal antibody (Ortho Pharmaceutical Corp) and incubated at 4 °C for 30 minutes The mixture was further incubated with an equal volume of 1:3 diluted rabbit serum (Lot 029957A, Behringwerke AG, Marburg, West Germany) as a source of complement at 37 °C for 45 minutes. This monoclonal antibody plus complement treatment was repeated Treated cells were washed once and adjusted to an appropriate number of viable cells Complement-mediated cell lysis was confirmed by trypan blue dye exclusion and cytofluorography. Eighty-eight percent to 95% killing of each T-cell subset occurred following treatment with the corresponding antibody and complement. OKT8-treated T cells were enriched for OKT4-positive cells and OKT4-treated T cells for OKT8-positive cells. These isolated T-cell subsets were referred to OKT4$^+$ and OKT8$^+$ cells, respectively, and were used as responder cells in AMLR.

Preparation of non-T, nonphagocytic PBMC. For the separation of nonphagocytic cells, 5% silica particle solution (KAC-2, Japan Immunoresearch Laboratory, Takasaki, Japan) was added to heparinized peripheral blood at an 1:9 ratio and incubated at 37°C for 60 minutes The mixture was then layered over a Ficoll-Hypaque density gradient and centrifuged at 400 × G for 30 minutes Furthermore, T cells were removed from this nonphagocytic cell fraction by using the rosette sedimentation technique.[19] The resulting non-T, nonphagocytic PBMC were approximately 10% positive for nonspecific esterase staining and contained ≤9% OKT3-positive and ≤15% Ia-positive cells, respectively. These cells were cryopreserved[20] until the use for assaying BFU-E.

Autologous mixed lymphocyte reaction (AMLR). A bulk AMLR was performed by culturing 5×10^6 T cells with 5×10^6 irradiated (20Gy) autologous non-T cells in 10 mL of RPMI (GIBCO, Grand Island, NY) supplemented with 15% pooled human serum at 37°C in a humid 5% CO_2 atmosphere.[21] For unstimulated cultures, 5×10^6 irradiated autologous T cells were added instead of non-T cells. In some experiments, OKT4$^+$ or OKT8$^+$ cells were used as responder cells Responder cells were harvested on days 3, 7, and 12 of AMLR and T cells were separated again by using the rosette sedimentation technique.[19] These responder T cells were washed three times with RPMI 1640 before coculturing with non-T, nonphagocytic autologous PBMC for BFU-E assays

Treatment with OKIal monoclonal antibody and radiation In some experiments, responder T cells harvested from AMLR (1×10^7/mL) were treated with an OKIal monoclonal antibody (50

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Table 1. Effect of T Cells Activated by Autologous MLR (AMLR) on BFU-E Growth

Type of T Cells Added*	No. of BFU-E /2 × 10^5 Cells	% of Control BFU-E‡
Control (No cells added)	77 ± 20 †	100
T/Tx from day 3 AMLR	83 ± 17	114 ± 9 8
T/non-Tx from day 3 AMLR	110 ± 21	164 ± 21 0
Control (No cells added)	94 ± 16 †	100
T/Tx from day 7 AMLR	96 ± 16	102 ± 1.5
T/non-Tx from day 7 AMLR	53 ± 19	50 ± 12 0
Control (No cells added)	113 ± 32 †	100
T/Tx from day 12 AMLR	124 ± 31	111 ± 5 2
T/non-Tx from day 12 AMLR	64 ± 25	50 ± 11.3

T cells stimulated by autologous T cells or non-T cells were harvested from day 3, 7, or 12 AMLR 1 × 10^5 AMLR-activated T cells were cocultured with 2 × 10^5 autologous non-T, non-phagocytic PBMC for assaying BFU-E. Each value represents M ± SEM in 5 experiments

*T/Tx and T/non-Tx indicate the combination of T cells and irradiated T cells or non-T cells in AMLR, respectively.

†Statistically significant at $P < 0\ 025$ level.

‡Percent of control BFU-E–derived colonies was calculated as follows (No of BFU-E colonies in coculture with AMLR-activated T cells/No. of BFU-E colonies in culture with no added T cells) × 100.

μg/mL; Ortho Pharmaceutical Corp) and complement before cocultures as described above. Treated cells were washed once and resuspended in the initial volume. Radiation treatment was performed by 20 Gy irradiation of the AMLR-activated T cells.

BFU-E assays. BFU-E were assayed by using the technique of Iscove in methylcellulose method[22] with a minor modification. Non-T, nonphagocytic PBMC (2 × 10^5/plate) with or without AMLR-activated T cells were cultured in 0 9% mehtylcellulose in Iscove's modified Dulbecco's medium (Sigma Chemical Co, St Louis, Mo) supplemented with 30% fetal calf serum, 1 unit of erythropoietin (Connaught Step III, lots 3083-1, 3084-1), and 5% PHA-stimulated leukocyte-conditioned medium[23] as a source of burst-promoting activity Cultures were performed in triplicate in 35 mm plastic dishes (Falcon #3010, Oxnard, Calif) and maintained at 37 °C in 5% CO_2 atmosphere with 100% humidity. On day 14 colonies with more than 100 hemoglobinized cells or clusters of three or more subcolonies were scored as BFU-E. Colonies were confirmed as erythroid by staining for hemoglobin with benzidine and by May-Giemsa staining.

Statistical analysis. The Student *t* test was employed for statistical analysis of the data obtained in the present study.

RESULTS

Table 1 shows effects of AMLR-activated T cells on in vitro BFU-E growth. When T cells stimulated by irradiated autologous T cells (T/Tx) were added to autologous non-T, nonphagocytic PBMC for BFU-E assays, they did not affect BFU-E growth. T cells activated by irradiated autologous non-T cells (T/non-Tx) increased BFU-E growth at a significant level ($P < 0.025$) when they were harvested on day 3 of AMLR. In contrast, T cells activated by autologous non-T cells for 7 or 12 days in AMLR showed a significant suppression of BFU-E growth ($P < 0.025$) A dose-dependent enhancement or suppression of BFU-E growth was observed. In a representative experiment, percentages of control BFU-E–derived colonies were 117, 160, and 167 with the addition of 0.5 × 10^5, 1.0 × 10^5, and 2.0 × 10^5 day 3 AMLR-activated T cells, respectively, while they were 62, 54, and 27 with the addition of 0.5 × 10^5, 1.0 × 10^5, and 2.0 × 10^5 day 7 AMLR-activated T cells, respectively.

Table 2 provides the data to determine whether AMLR-activated T cells will affect allogeneic BFU-E growth or not. Activated T cells harvested from day 3 AMLR showed a significant increase ($P < 0.05$) of not only autologous but also allogeneic BFU-E growth when they were added to BFU-E assays. However, activated T cells harvested from day 7 AMLR suppressed only autologous BFU-E growth ($P < 0.05$). Allogeneic BFU-E growth was unaffected by adding day 7 AMLR-activated T cells to BFU-E assays

Next, effects of AMLR-activated OKT4$^+$ or OKT8$^+$ cells on BFU-E growth were studied. As shown in Fig 1, OKT4$^+$ cells activated by autologous non-T cells exerted regulatory functions while AMLR-activated OKT8$^+$ cells did not. Activated OKT4$^+$ cells harvested from day 3 AMLR increased BFU-E growth in comparison with the unactivated control ($P < 0.025$). This increase in BFU-E growth was comparable to that exerted by day 3 AMLR-activated T cells. In contrast, activated OKT4$^+$ cells harvested from day 7 AMLR showed a significant suppression of BFU-E growth ($P < 0.025$), which was almost identical to that exerted by day 7 AMLR-activated T cells.

AMLR-activated T cells expressing regulatory functions for BFU-E growth were studied for the presence of Ia antigens and radiosensitivity. When AMLR-activated T

Table 2. Effect of AMLR-Activated T Cells on Autologous or Allogeneic BFU-E

Type of T Cells Added*	PBMC Cultured for BFU-E Assay	No of BFU-E/2 × 10^5 Cells	% of§ Control BFU-E
Control (No cells added)	autologous	72 ± 24 †	100
T/non-Tx from day 3 AMLR		132 ± 31	156 ± 7.5
Control (No cells added)	allogeneic	74 ± 25 †	100
T/non-Tx from day 3 AMLR		128 ± 31	156 ± 6.3
Control (No cells added)	autologous	77 ± 30 †	100
T/non-Tx from day 7 AMLR		37 ± 8	49 ± 6.4
Control (No cells added)	allogeneic	116 ± 45 ‡	100
T/non-Tx from day 7 AMLR		115 ± 48	94 ± 8 5

1 × 10^5 AMLR-activated T cells were cocultured with 2 × 10^5 autologous or allogeneic non-T, nonphagocytic PBMC for assaying BFU-E. Each value represents M ± SEM in 4 experiments.

*See footnote in Table 1.

†Statistically significant at $P < 0.05$ level.

‡Not significant.

§See footnote in Table 1

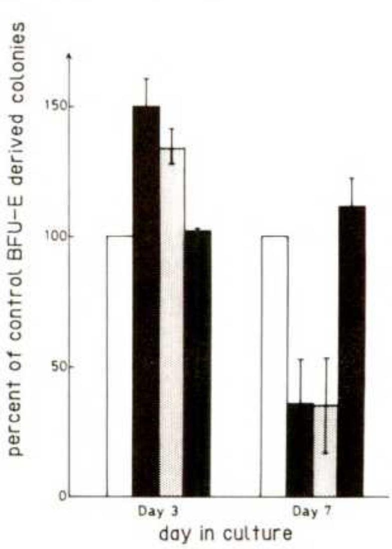

Fig 1. Effect of OKT4$^+$ cells and OKT8$^+$ cells activated by AMLR on BFU-E growth. OKT4$^+$ or OKT8$^+$ T cells were cultured with autologous non-T cells in AMLR. 1×10^5 AMLR-activated OKT4$^+$ or OKT8$^+$ cell were harvested on day 3 or day 7 and cocultured with 2×10^5 autologous non-T, nonphagocytic PBMC for assaying BFU-E. For percent of control BFU-E–derived colonies, see footnote in Table 1. Data indicate M ± SEM in 5 experiments. □, Control; ■, AMLR activated T cells added; ░, AMLR activated OKT4$^+$ cells added; ■, AMLR activated OKT8$^+$ cells added.

cells were treated with an OKIal monoclonal antibody and complement before cocultures, both BFU-E enhancing and suppressing activities were completely abolished (Fig 2). Enhancement and suppression of BFU-E growth were unaffected by addition of AMLR-activated T cells treated with complement alone. Similarly, both of the two opposing functions were completely abolished when these activated T cells were 20 Gy irradiated before cocultures (Fig 3).

When T cells were stimulated by allogeneic non-T cells instead of autologous ones in a conventional one-way MLR, T cells activated by alloantigens for 3 or 7 days in MLR had no effects on in vitro growth of BFU-E as shown in Fig 4. On

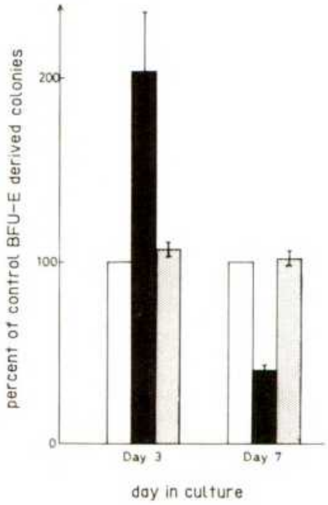

Fig 2. Effect of OKIal plus complement treatment on AMLR-activated T cells in the regulation of BFU-E growth. 1×10^5 AMLR-activated T cells were treated with complement alone or OKIal monoclonal antibody and complement before culturing with 2×10^5 autologous non-T, nonphagocytic PBMC for assaying BFU-E. For percent of control BFU-E–derived colonies, see footnote in Table 1. Data indicate M ± SEM in 3 experiments. □, Control; ■, AMLR activated T cells + C'; ░, AMLR activated T cells + OKIal + C'.

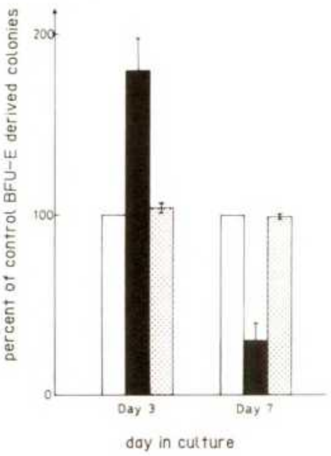

Fig 3. Effect of radiation on AMLR activated T cells in the regulation of BFU-E growth. 1×10^5 AMLR activated T cells were 2,000 rad irradiated before culturing with 2×10^5 autologous non-T, nonphagocytic PBMC for assaying BFU-E. For percent of control BFU-E–derived colonies, see footnote in Table 1. Data indicate M ± SEM in 4 experiments. □, Control; ■, nonirradiated AMLR activated T cells added; ░ irradiated AMLR activated T cells added.

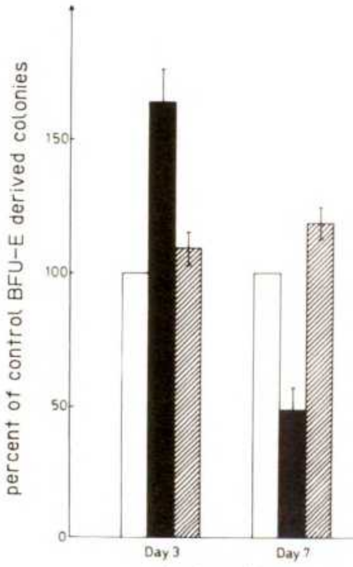

Fig 4. Effect of T cells activated by alloantigens in MLR on BFU-E growth. 1×10^5 alloantigen-activated T cells or AMLR-activated T cells were cocultured with 2×10^5 autologous non-T, nonphagocytic PBMC for assaying BFU-E. For percent of control BFU-E–derived colonies, see footnote in Table 1. Data indicate M ± SEM in 8 experiments for the day 3 harvest and 6 experiments for the day 7 harvest. □, Control; ■, AMLR activated T cells added; ▨, MLR activated T cells added.

the other hand, T cells activated by AMLR expressed dual functions for BFU-E growth depending on the culture period of AMLR.

DISCUSSION

The present study demonstrates that T cells activated by autologous non-T cells in AMLR exert both enhancing and suppressing activities in the regulation of in vitro erythropoiesis; those from day 3 AMLR enhanced and those from days 7–12 AMLR suppressed BFU-E growth. Both of these regulatory functions were mediated by an Ia-positive and radiosensitive population within the AMLR-activated OKT4$^+$ cells. Furthermore, activated T cells harvested at an early stage of AMLR enhanced both autologous and allogeneic BFU-E growth; those at a late stage of AMLR suppressed only autologous BFU-E. These observations suggest that in vitro differentiation and proliferation of erythroid progenitor cells may be regulated at least partly by autoreactive T cells.

Several investigators have shown that in vitro growth of erythroid progenitor cells can be enhanced by coculturing them with normal or unactivated T cells and T-cell subsets.[1-3] Similarly, recent studies indicate that unactivated T-cell subsets, both OKT4$^+$ and OKT8$^+$ cells, could stimulate in vitro growth of BFU-E without collaboration between them.[24,25] In the present study, only OKT4-positive T cells activated by 3-day AMLR increased in vitro growth of peripheral blood BFU-E. These AMLR-induced BFU-E–enhancing T cells were found to be Ia-positive and radiosensitive. Therefore, this AMLR-activated T-cell subset is probably different from T-cell subsets affecting BFU-E growth reported by others.[2,3,23,24] Mechanisms by which AMLR-activated T cells exerted the BFU-E growth-enhancing activity are unknown. One possible explanation is that they are capable of producing burst-promoting activities, as described by Wisniewski et al.[25]

The present study provides evidence suggesting that AMLR-activated T cells may exert dual functions in the regulation of BFU-E growth. Torok-Storb et al[2,26] have also demonstrated that normal T cells affecting BFU-E growth can be divided into at least two functionally different subsets defined by monoclonal antibodies and that a population of Ia-positive T cells limits BFU-E growth. Similarly, Mangan et al[3] reported the presence of BFU-E–regulating T-cell subpopulations defined by Fc receptors and monoclonal antibodies. However, these T-cell subsets with regulatory functions were not activated in vitro. Our data show that in vitro colony formation by BFU-E was affected by the two functionally different T-cell subsets. Both of them were Ia-positive and radiosensitive within the OKT4$^+$ phenotype but they were harvested at different stages of AMLR for activation. From our data, however, it is not determined whether these T-cell subsets with opposing functions were identical or not before activation of AMLR; one possibility is that there may exist two distinct subpopulations before activation, and another is that the same T-cell population may be induced to express the two opposing functions successively upon activation in AMLR.

In contrast to nonspecific or genetically unrestricted stimulation of BFU-E growth by unactivated or activated T cells in our study and others,[1-3,24,25] T cells activated by 7-day AMLR suppressed only autologous, but not allogeneic BFU-E. This suggests a possible presence of genetic restriction in expressing a regulatory function by AMLR-induced BFU-E–suppressor T cells. Genetic restriction of T cells suppressing erythroid colony growth has been reported by Torok-Storb and Hansen[26] and Lipton et al.[27] These erythroid suppressor T cells were Ia-positive, although they were not activated in vitro. Data from the former study[26] suggest that a subset of Ia-positive T cells may suppress BFU-E growth by limiting a monocyte-dependent T-cell stimulation activity for BFU-E growth through HLA-DR antigen recognition.[28] This is unlikely in our study since OKIal plus complement treatment of AMLR-activated T cells abolished suppression of BFU-E growth but did not result in increased stimulation of BFU-E growth. Since activated T cells are able to generate a variety of lymphokines,[11,29,30] and interferons can inhibit proliferation of hematopoietic progenitor cells,[11,31] it is possible that suppression of BFU-E growth may be mediated by interferon(s) produced by activated OKT4$^+$ cells from day 7 AMLR. Recently we have shown that concanavalin-A–stimulated T cells can suppress in vitro growth of erythroid and myeloid progenitor cells[6]; they were OKT8-positive but Ia-negative. Thus, our data together with others indicate that T cells involved in the regulation of in vitro erythropoiesis are heterogenous in their immunologic phenotype.

AMLR has been shown to be abnormal or defective in a variety of diseases characterized by disturbed immune regulations.[18,32] Recently Kotani et al[33] have demonstrated that OKT4$^+$ cells activated for 3 days in AMLR exert helper activity for pokeweed mitogen-induced immunoglobulin synthesis while those from day 7 AMLR express suppressor activity. These observations suggest that AMLR may play an important role in maintaining normal immune regulation. In this regard, our data indicate a possibility that AMLR may be actively involved in the immune-mediated regulation of in vitro erythropoiesis; dual functions successively exerted by AMLR-activated T cells could provide a "regulatory circuit" in which AMLR-induced BFU-E–suppressor T cells may exert a "feedback suppression" for BFU-E growth stimulated by AMLR-induced BFU-E–enhancing T cells.

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Controlled Trial of Desmopressin in Liver Cirrhosis and Other Conditions Associated With a Prolonged Bleeding Time

By Pier Mannuccio Mannucci, Vicente Vicente, Luciana Vianello, Marco Cattaneo, Ignacio Alberca, Maria Piera Coccato, Elena Faioni, and Daniela Mari

The synthetic vasopressin derivative desmopressin (DDAVP) shortens a prolonged bleeding time (BT) in patients with uremia, congenital platelet dysfunction, and von Willebrand disease. To establish the limits of the clinical usefulness of DDAVP, a controlled randomized study was carried out in 53 patients and ten volunteers with different conditions that have in common a prolonged BT. DDAVP significantly shortened the BT in 21 cirrhotics ($P < .01$), in eight patients with unclassified prolonged BT ($P < .05$) and in ten volunteers taking the antiplatelet drugs aspirin ($P < .05$) and ticlopidine. The BT changes were not statistically significant in 15 patients with severe thrombocytopenia nor in nine with congenital platelet dysfunction, even though a few patients with storage pool deficiency responded with a marked BT shortening. Our findings indicate that DDAVP might be given when biopsies or other surgical procedures must be carried out in patients with prolonged BT. However, the compound is often ineffective in patients with thrombocytopenia or congenital platelet dysfunction.

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RECENT STUDIES have shown that the synthetic vasopressin derivative desmopressin (DDAVP) shortens the prolonged bleeding times (BT) associated with a variety of clinical disorders of primary hemostasis.[1-4] In von Willebrand disease, the most common congenital cause of prolonged BT, the effectiveness of DDAVP is clearly explained by the fact that the prolonged BT is due to low or abnormal plasma von Willebrand factor (vWF), the most important determinant of platelet adhesion to the damaged vessel wall.[5] By releasing autologous vWF from storage sites (possibly the endothelial cells), DDAVP transiently normalizes vWF levels and shortens the BT, at least in patients with vWF that is functionally normal.[1] However, DDAVP also shortens the BT in such conditions as uremia[2,3] and congenital and acquired platelet dysfunctions,[4] in which no quantitative or qualitative vWF abnormality has been convincingly demonstrated. These observations, together with a recent in vitro study demonstrating a local hemostatic effect of the drug on the vessel wall,[6] led us to postulate that DDAVP might have broader clinical usefulness as a primary hemostatic agent in patients with prolonged BT due to causes other than low or dysfunctional vWF. To establish the limits of the clinical usefulness of DDAVP, we have carried out a controlled study in 53 patients with different clinical conditions that have a prolonged BT in common, and in ten volunteers taking the antiplatelet drugs aspirin or ticlopidine.

MATERIALS AND METHODS

Patients. Fifty-three patients (25 men and 28 women, with a median age of 37 years; range, 15 to 65) seen at the A. Bianchi Bonomi Hemophilia and Thrombosis Center in Milan, Italy, or at the Department of Hematology in Salamanca, Spain, were admitted to the study on the basis of a BT of ten minutes or longer when measured before the first experimental treatment (see below). Even though the upper normal BT limit is seven minutes at both centers, an admission limit of ten minutes was chosen because we consider this value to be the clinical threshold between normal and abnormal hemostasis. Of the 53 patients admitted to the study, 21 had alcoholic or postnecrotic liver cirrhosis, diagnosed by liver tests and biopsy. Their platelet counts were normal or moderately low (median, 106 × 10^9/L; range, 45 to 286). Fifteen patients had severe thrombocytopenia (median platelet count, 13 × 10^9/L; range, 1 to 53), due to increased platelet destruction by autoantibodies in five cases and due to decreased bone marrow production in ten cases (seven had aplasia, one familial thrombocytopenia, one myeloma, one chronic lymphocytic leukemia). Nine patients belonging to different kindreds had congenital platelet dysfunction, diagnosed as delta storage pool deficiency in seven and Glanzmann thrombasthenia in two cases, according to previously described criteria.[7,8] Finally, eight patients had prolonged BT associated with lifelong histories of bleeding but with normal platelet counts, no laboratory evidence of von Willebrand's disease, no defect in platelet aggregation or secretion, no storage pool deficiency, nor any underlying condition known to be associated with a prolonged BT. These unexplained prolonged BT might be due to vascular defects or to as yet unidentified platelet dysfunctions. We have called this patient group "unclassified prolonged BT." None of the patients had taken aspirin or other drugs that affect platelet behavior for at least ten days before the study.

We also studied six volunteers who had borderline or slightly prolonged BT due to the ingestion of 500 mg aspirin two hours before the study, and four volunteers who had very prolonged BT because they had taken a daily dose (500 mg) of the antiplatelet drug ticlopidine for six days before the study. Ticlopidine is a potent antiplatelet agent, available in Europe and presently under clinical trials in the United States and in Canada, which markedly prolongs the BT through a presently unknown mechanism.[9] All the patients and volunteers were aware of the nature and the purpose of the study, and all gave informed consent according to the Declaration of Helsinki.

Design of the study. Patients and volunteers were randomly assigned to a single infusion of DDAVP (Valeas, Milan, Italy) or of saline, and each of them was crossed over from one treatment to the other after 20 days. After a rest period of at least 30 minutes, 0.3 μg of DDAVP per kilogram body weight was added to 50 mL of physiological saline and infused over a period of 30 minutes; physiological saline alone was infused in the same way. The study

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could not be performed on a double-blind basis because DDAVP infusion can be detected by an accompanying mild facial flushing.

Blood samples were collected and plasma prepared as previously described.[1]

Laboratory methods. The tests were performed in Milan and Salamanca with the same reagents, methods, and standards, except that vWF multimeric analysis was done in Milan on plasma samples kept frozen at −70 °C and transported in dry ice. All tests were carried out on blood drawn before the infusion of DDAVP or saline (time 0), immediately after the infusion (time 30), and four hours after the infusion had been started (time 240).

Bleeding times were obtained with the Simplate II device (General Diagnostics, Milan, Italy). Results were expressed as the average BT from the two vertical incisions. In both laboratories, the range of normal with this method was three to seven minutes. Platelets were counted by phase-contrast microscopy. The prothrombin time (PT) was determined by using the Manchester Comparative Reagent (overseas version, Laboratori Baldacci, Pisa, Italy), and the activated partial thromboplastin time (APTT), with a commercial reagent (Automated PTT, General Diagnostics). Values of both the coagulation screening tests were expressed as ratios of patient to pooled normal plasma (obtained from 15 healthy women and 15 healthy men). Factor VIII (FVIII) was assayed by a one-stage clotting method based on the APTT.[1,3] vWF antigen (vWF:Ag) was assayed by quantitative immunoelectrophoresis, using a commercial monospecific antiserum (Istituto Behring, Scopitto, Aquila).[1,3] Ristocetin cofactor (RiCof) was assayed with formalin-fixed platelets.[1,3] FVIII, vWF:Ag, and RiCof were expressed in units per deciliter, with reference to pooled normal plasma calibrated against the First International Plasma Standard for Factor VIII-related Activities (National Institute for Biological Standards, London). vWF multimers were analyzed by agarose electrophoresis in the presence of sodium dodecyl sulfate (SDS), as previously described.[1]

Statistical analysis. The BT values were not normally distributed, and there were BT that did not stop after 30 minutes and were recorded as longer than 30 minutes. Hence BT values before and after DDAVP or placebo were expressed as medians and ranges. The other measurements were normally distributed, and thus were expressed as means ± SD. Correlation coefficients were calculated either by the Spearman *rho* test or as linear correlation coefficients (*r*).

RESULTS

An overall picture of the BT (given as medians and ranges) before and after DDAVP or saline in the patient and volunteer groups is given in Table 1. The two median BT obtained before either treatment (time 0) did not differ significantly in any of the groups.

In patients with liver cirrhosis, the prolongation of the baseline BT was unrelated to the platelet count (Fig 1) (no platelet function tests were performed). At 30 and 240 minutes after DDAVP, the median BT was significantly shorter than at time 0 ($P < .01$, Table 1). In nine of 21 patients at time 30 and in two at time 240, the BT had become shorter than ten minutes, the limit for admission to the study. Responses or non-responses to DDAVP occurred irrespective of the baseline platelet count. After saline, the median BT had not changed significantly at time 30 and at time 240 (Table 1). The BT shortened to less than ten minutes in two patients at time 30 and in five at time 240 (Fig 1). In patients with unclassified prolonged BT, the median BT became significantly shorter after DDAVP ($P < .05$ Table 1); in four of eight patients BT became shorter than ten minutes at time 30 and in three at time 240, but in none after saline (Fig 2).

In patients with thrombocytopenia, there was no significant change in the BT after either saline or DDAVP (Table 1), and the BT was shortened to less than ten minutes in only one case after saline. In the two patients with Glanzmann thrombasthenia, the baseline BT was longer than 30 minutes and remained so at all times after DDAVP or saline. In the seven patients with congenital storage pool deficiency, the median BT did not significantly change after DDAVP or saline (Table 1). After DDAVP, the BT became shorter than ten minutes in two patients at time 30 and in one at time 240, but in none after saline (Fig 3).

Table 1. Bleeding Times Before and After DDAVP or Saline Infusion in Patients With Prolonged Bleeding Times

	DDAVP			Saline		
Group	Time 0 (Before Infusion)	Time 30 (30 min After Infusion)	Time 240 (4 h After Infusion)	Time 0 (Before Infusion)	Time 30 (30 min After Infusion)	Time 240 (4 h After Infusion)
Liver cirrhosis (n = 21)	16 (10-30)	12** (6-20)	13** (5-22)	15 (9-30)	14 (8-25)	14 (7-29)
Thrombocytopenia (n = 15)	>30 (17->30)	>30 (12->30)	>30 (11->30)	>30 (12->30)	>30 (10->30)	>30 (8->30)
Storage pool deficiency (n = 7)	19 (11->30)	11 (7->30)	24 (8->30)	25 (11->30)	30 (10->30)	24 (11->30)
Unclassified prolonged bleeding time (n = 8)	17 (10->30)	11* (7->30)	12* (7->30)	16 (10->30)	16 (10->30)	15 (10->30)
Aspirin takers (n = 6)	8 (7-10)	6** (5-8)	6** (5-7)	8 (7-9)	7 (5-9)	8 (7-10)
Ticlopidine takers (n = 4)	24 (14->30)	8 (6-9)	19 (9-19)	13 (12->30)	17 (14-21)	15 (11-22)

Figures indicate median values and, in parentheses, ranges. Asterisks indicate significance of difference between post-DDAVP or -saline values and baseline values (*$P < .05$, **$P < .01$). Significance was not evaluated for the ticlopidine group because of the small number of patients.

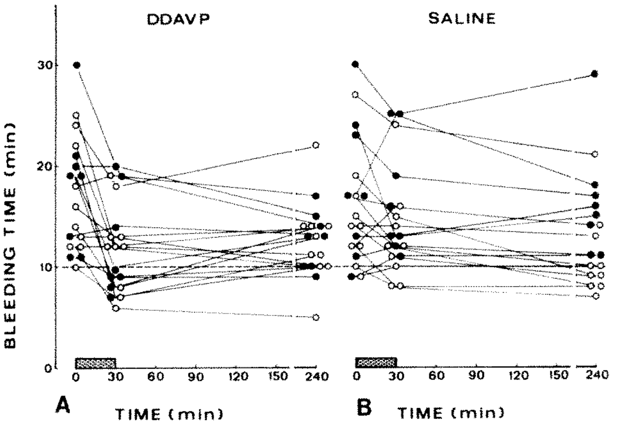

Fig 1. **Changes in bleeding times before (time 0), immediately after (time 30), and 4 hours after (time 240) infusion of DDAVP (left panel) or saline (right panel) in 21 patients with liver cirrhosis. Patients with baseline platelet counts higher than 100 × 10^9/L are indicated by open circles; patients with platelet counts lower than 100 × 10^9/L, by solid circles. The dashed horizontal line indicates a bleeding time of 10 minutes, the value for admission to the trial.**

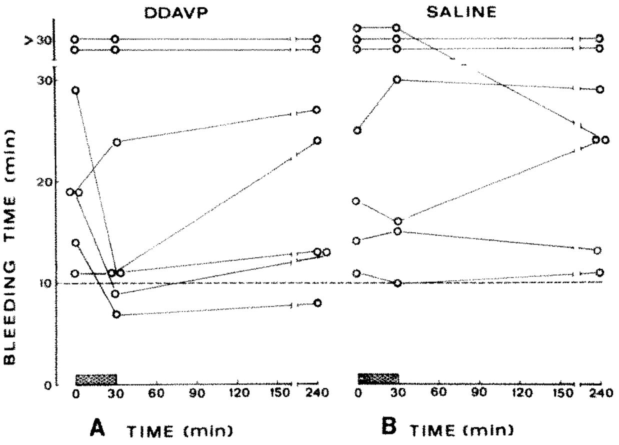

Fig 3. **Changes in bleeding times in seven patients with delta storage pool deficiency.**

In the six volunteers who took aspirin the median BT was significantly shortened at times 30 and 240 after DDAVP ($P < 0.05$) but not after saline (Table 1 and Fig 4). Facial flushing occurred in all cases after DDAVP, whether or not they had taken aspirin. In all four volunteers who took ticlopidine, whose baseline BT were longer than those taking aspirin (Fig 4), the BT had shortened to less than ten minutes at time 30 after DDAVP but had returned to the prolonged values in three at time 240. At no time after saline did the BT shorten to ten minutes or less. Flushing occurred in all cases after DDAVP, whether or not they had taken ticlopidine. These data were not statistically analyzed because of the small number of patients.

After DDAVP or saline, all measurements other than the BT behaved in the same way in each patient or volunteer group. Hence the values for all groups were pooled and analyzed together (Table 2). After DDAVP, there were significant increases in FVIII, vWF:Ag, RiCof ($P < .001$) at times 30 and 240; shortening of the APTT, expressed by a lower ratio ($P < .001$), at times 30 and 240; slight prolongation of the PT expressed by a higher ratio ($P < .01$) at times 30 and 240; and no significant change in the platelet counts. No significant change in any of these was seen after saline. Correlations between the DDAVP-induced changes (differences between pretreatment and posttreatment values) in BT and in other measurements were calculated both for each patient group and for all the groups considered as a whole. No significant correlations were found (data not shown).

The multimeric structure of vWF before and after DDAVP was studied in at least two patients in each group, comparing, when available, one patient in whom the BT became shorter than ten minutes and another in whom it did not. Before DDAVP, the multimeric structure in the patients' plasma was no different from that observed in normal plasma. After DDAVP, larger vWF multimers than those present in pretreatment plasma appeared at time 30 in all patients, whether or not the BT became shorter (Fig 5). There were no changes in the multimeric structure when the patients were treated with saline.

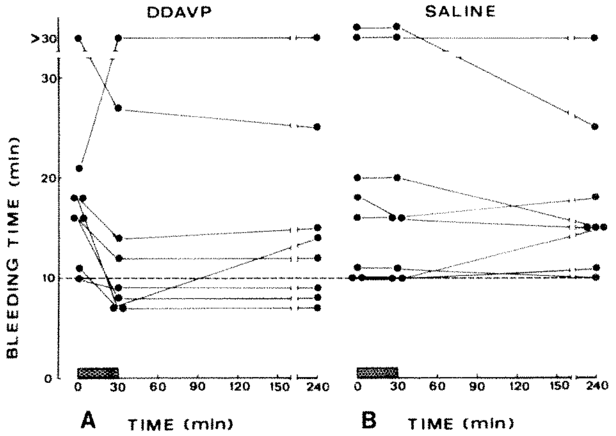

Fig 2. **Changes in bleeding times in eight patients with unclassified prolonged bleeding time.**

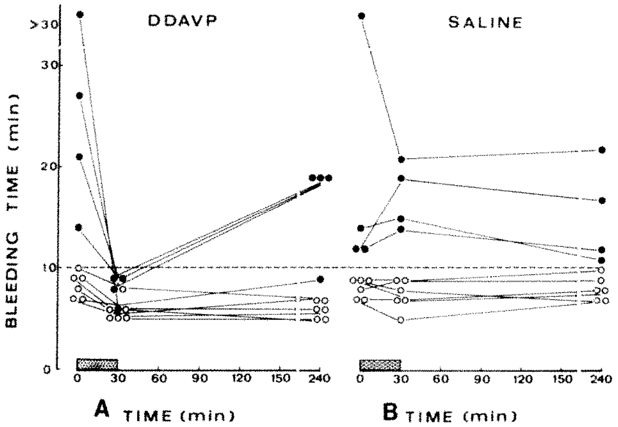

Fig 4. **Changes in bleeding times in six volunteers taking 500 mg aspirin (open circles) and in four volunteers taking 500 mg ticlopidine (solid circles).**

Table 2. Laboratory Measurements Before and After DDAVP or Saline Infusion

	DDAVP			Saline		
	Time 0 (Before Infusion)	Time 30 (30 min After Infusion)	Time 240 (4 h After Infusion)	Time 0 (Before Infusion)	Time 30 (30 min After Infusion)	Time 240 (4 h After Infusion)
vWF:Ag (U/dL)	252 ± 137	291 ± 112***	339 ± 126***	247 ± 129	247 ± 123	249 ± 126
RiCof (U/dL)	215 ± 168	267 ± 150***	312 ± 169***	220 ± 172	213 ± 160	216 ± 164
FVIII (U/dL)	151 ± 59	255 ± 69***	310 ± 140***	146 ± 62	143 ± 60	147 ± 58
PT ratio	1.10 ± 0.14	1.13 ± 0.15**	1.13 ± 0.15**	1.09 ± 0.12	1.10 ± 0.12	1.09 ± 0.12
APTT ratio	1.11 ± 0.25	1.02 ± 0.26***	1.00 ± 0.25***	1.11 ± 0.25	1.12 ± 0.26	1.10 ± 0.24
Platelet count ($\times 10^9$/L)	143 ± 122	138 ± 117	140 ± 129	143 ± 116	140 ± 114	152 ± 122

Figures indicate means ± SD. Asterisks indicate significance of differences between post-DDAVP or -saline values and baseline values: $^{**}P < .01$; $^{***}P < .001$ (*t* test for paired samples).

DISCUSSION

The purpose of this controlled study was to see whether or not DDAVP is effective in a number of clinical situations that all have prolonged BT, the best available laboratory indication of abnormal primary hemostasis. The most significant finding was that after DDAVP, the BT became less than ten minutes in about half of the cirrhotic patients. This is the value we consider the upper clinical threshold for normal primary hemostasis. DDAVP was also effective in patients with unclassified prolonged BT and in volunteers taking the antiplatelet drugs aspirin and ticlopidine, whereas the shortening of the BT was not significant in severe thrombocytopenia or in congenital platelet dysfunction, even though three of seven patients with congenital storage pool deficiency responded to DDAVP with a marked BT shortening.

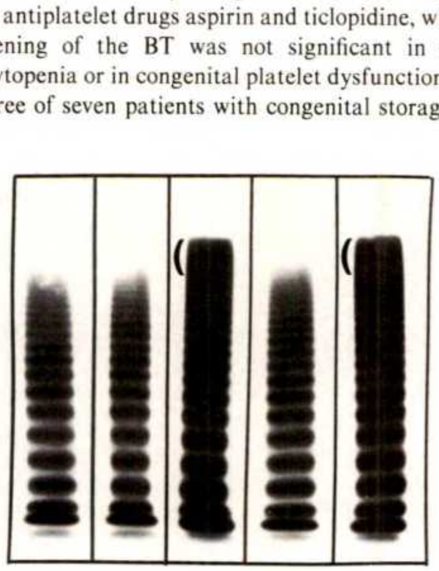

Fig 5. Autoradiograph pattern of plasma vWF electrophoresed in 1.4% high-gelling temperature agarose in the presence of SDS and detected by ^{125}I-labeled affinity-purified antibody. The arrow indicates the origin of the running gel and the anode is at the bottom of the gel. From left to right: normal plasma; plasma from a patient with liver cirrhosis before (bleeding time: 21 minutes) and 30 minutes after (bleeding time: 9 minutes) DDAVP; plasma from another patient with liver cirrhosis before (bleeding time: 20 minutes) and 30 minutes after (bleeding time: 20 minutes) DDAVP. The brackets indicate the large vWF multimers that appear in both plasma after DDAVP.

Why the BT is prolonged in cirrhotics is not completely understood.[10,11] Mild or moderately severe thrombocytopenia is usually present in cirrhosis and was also found in our patients, but their platelet counts were not negatively correlated with their BT. An abnormal vWF is also unlikely because patients had normal or high baseline levels of vWF:Ag and RiCof and the multimeric structure of vWF in their plasma did not differ from that of healthy subjects. Notwithstanding the present uncertainty about the causes of prolonged BT in cirrhotics, the shortening of the BT induced in about half of our patients by DDAVP indicates that this drug might be given when liver biopsies or other surgical procedures would be otherwise contraindicated by a prolonged BT. It must be emphasized, however, that the hemostatic derangement of liver disease is complex, and that the modest and short-lived shortening of the BT seen in most of the responders may not be sufficient to significantly reduce the clinical risk of hemorrhage. The APTT shortened after DDAVP but, at variance with others,[12] we have seen no shortening of the prothrombin time. Hence the hemostatic derangement of liver disease is not completely corrected by DDAVP, and only clinical experience will establish the hemostatic efficacy of the compound.

DDAVP also shortened the prolonged BT induced by the antiplatelet drugs ticlopidine and aspirin, the latter observation being in agreement with that made by Kobrinsky et al[4] in their study of two aspirin-treated patients. It also was useful in the group of patients with prolonged BT who had no alterations in platelet function tests that could explain their deranged primary hemostasis (vascular abnormalities? unidentified platelet dysfunction?). As for cirrhotics, these effects of DDAVP might be exploited when surgical procedures must be carried out and a shortening of the BT must be obtained rapidly, but clinical experience is needed to validate this hypothesis. There is little justification for giving DDAVP to patients with thrombasthenia, in agreement with Kobrinsky et al,[4] or to those with severe thrombocytopenia because the BT was not shortened. Unlike Kobrinsky, we did not find a significant shortening of the BT in patients with congenital storage pool deficiency. However, the analysis of the responses of individual patients indicates that the BT became shorter than ten minutes in two and shortened from 29 to 11 minutes in one, even though it remained prolonged in the remaining four (Fig 3). Hence it appears that in storage pool deficiency there are both responders and nonresponders, and that the response cannot be predicted on the basis of criteria such as the degree of baseline BT prolongation (Fig 3) and platelet levels of serotonin and adenosine diphosphate (data not shown).

Even though this controlled study establishes more clearly the therapeutic indications and limitations of DDAVP in disorders of primary hemostasis, the mechanism of the action of the drug is still unknown. Its poor efficacy in severe thrombocytopenia indicates that some critical platelet number is necessary for the drug to be effective. Poor efficacy in thrombasthenia and, to a lesser extent, congenital storage pool deficiency would apparently indicate that the platelets must also function normally. On the other hand, DDAVP was efficacious in some but not all patients with congenital storage pool deficiency, in volunteers taking antiplatelet drugs such as aspirin and ticlopidine, both of which, and particularly the latter,[9,13] induce severe platelet dysfunction. Even though drug-induced inhibition of vascular prostacyclin generation shortens the BT in rabbits,[14] it is unlikely that, as suggested by others,[15] this is a major mechanism for the DDAVP shortening effect because DDAVP shortened the BT in volunteers treated with 500 mg aspirin, a dose high enough to abolish vascular prostacyclin production.[16,17] On the other hand, Belch et al[18] have previously shown that DDAVP stimulates the production of plasma prostacyclin, suggesting that prostacyclin might cause the facial flushing seen after intravenous administration of DDAVP. We[19] and others,[20] however, did not demonstrate any change of prostacyclin after DDAVP. In agreement with Brommer et al,[21] we have also found that aspirin intake did not abolish the flushing of the face seen after DDAVP. Hence prostacyclin has probably little to do with the effects and side effects of DDAVP. A recent study has shown that ticlopidine administration induces a thrombasthenic-like functional state in normal platelets.[12] It remains to be explained why DDAVP, which promptly shortened the prolonged BT in our volunteers taking ticlopidine, did not shorten the prolonged BT in congenital thrombasthenia.

We have previously postulated that large vWF multimers transiently released by DDAVP from cellular compartments might enhance platelet adhesion and potentiate primary hemostasis.[1] Even though this hypothesis has been subsequently corroborated by the finding of heightened platelet adhesion in normal volunteers after DDAVP,[22] additional factors must be advocated to explain the hemostatic effectiveness of DDAVP because although large vWF multimers were released in all the patients we studied, the BT was not shortened in all. Other biological changes induced by DDAVP were a shortening of the APTT, previously observed by others[4,23] and probably due to the high FVIII levels achieved; prothrombin time prolongation, probably due to plasma dilution after water retention induced by the antidiuretic effect of the drug[24]; whereas, unlike Kobrinsky,[4] we saw no significant fall of the platelet count. These changes, however, seem unlikely to provide clues for the effectiveness of DDAVP because they were not correlated with BT changes and occurred in all groups, regardless of the effectiveness of DDAVP on the BT.

NOTE ADDED IN PROOF

At the time this manuscript was accepted for publication, Burroughs et al (Br Med J 291:1377, 1985) showed that DDAVP significantly shortened bleeding time in cirrhotics.

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Responses of Hemopoietic Precursors to 13-cis Retinoic Acid and 1,25 Dihydroxyvitamin D_3 in the Myelodysplastic Syndromes

By Grant Swanson, Vincent Picozzi, Rodman Morgan, Frederick Hecht, and Peter Greenberg

To determine the effects of the "maturation-inducing" agents 13-cis retinoic acid and 1,25 dihydroxyvitamin D_3 on marrow cells from normal individuals and patients with myelodysplastic syndromes (MDS), we assessed marrow hemopoietic clonogenicity and differentiation response patterns to these agents. These vitamins caused increased proliferation in vitro of normal clonogenic marrow myeloid precursor cells (CFU-GM), decreased erythroid precursors (BFU-E), and no change in multipotent stem cells (CFU-GEMM). Marrow hemopoietic colony-forming cell incidence was generally subnormal in the 22 MDS patients evaluated. In vitro exposure to both agents caused various patterns of alteration of MDS hemopoietic colony and cluster formation, with similar but more pronounced effects evoked by retinoic acid. In the vast majority of MDS patients, enhanced marrow clonal granulocyte-monocyte differentiation and decreased BFU-E growth were noted after in vitro exposure to these vitamins. Correlation of biological effects was demonstrated between in vivo changes of peripheral neutrophil counts and in vitro responses of myeloid precursors for ten MDS patients treated with an eight-week therapeutic course of retinoic acid. Cytogenetic analyses indicated persisting aneuploidy or coexisting normal and aneuploid karyotypes in the cultured MDS myeloid cells and (with one exception) in native marrow cells from the treated patients. The varying responses of the MDS cells may monitor differing proportions of normal versus leukemic marrow cells susceptible to proliferative and differentiative expression on exposure to these agents.

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THE MYELODYSPLASTIC SYNDROMES (MDS) provide a clinical setting for the evaluation of a group of relatively indolent disorders with a propensity to evolve into acute myeloid leukemia (AML). Controversy exists regarding prospective categorization of these patients as preleukemic. In vitro marrow culture studies and marrow cytogenetics are biological parameters that have been useful in characterizing this group of patients with regard to their potential for blastic transformation and for survival.[1,2] These studies have also improved our understanding of possible pathogenetic mechanisms underlying these disorders. Current management of these patients during their chronic indolent phase has generally been limited to supportive measures. However, recent approaches to therapy have included the use of such "maturation-inducing" agents as retinoic acid, vitamin D, and low-dose cytosine arabinoside.[3–5] These treatment regimens are based on the concept that a critical lesion underlying leukemia relates to an uncoupling of proliferation and differentiation at the level of the hemopoietic stem cell.[6–10] Induction of differentiation in culture diminishes the ability of leukemic stem cells to self-generate in vitro and reduces in vivo leukemogenicity.[7,8,10,11] Studies with murine models have demonstrated increased survival of animals with induced leukemia after their in vivo treatment with humoral inducers of myeloid differentiation or vitamin D.[11,12]

Due to the reported effects of retinoic acid and vitamin D on the proliferative and differentiative potential of normal and leukemic cells in vitro,[13–18] we have further assessed the in vitro hemopoietic clonogenicity and differentiation patterns of normal and MDS marrow cells in response to these maturation-inducing agents. To determine whether biological alterations occur after in vivo treatment of MDS patients with retinoic acid, we also evaluated marrow cytogenetics and in vitro clonal responses to these agents before and after an eight-week clinical trial of oral 13-cis retinoic acid therapy.

MATERIALS AND METHODS

Colony formation by granulocyte–macrophage (CFU-GM), erythroid (BFU-E), and pluripotent (CFU-GEMM) progenitor cells. Marrow cells were obtained by aspiration after informed consent was given in accordance with guidelines established by the Stanford Human Experimentation Committee. The cells were washed and then suspended in Iscove's medium containing 15% fetal calf serum (FCS) (Hyclone, Logan, Utah). The buoyant (<1.077 g/cm^3) mononuclear marrow cells were procured after centrifugation through Hypaque-Ficoll. To determine CFU-GM, 1.5×10^5 marrow cells were plated in 35-mm Lux dishes in 1 mL of a mixture of Iscove's medium, 1.1% methylcellulose, 30% FCS, and 15% human placental conditioned medium (HPCM) as a source of colony-stimulating factor (CSF-GM), as previously described.[19,20] Myeloid colonies and clusters were generally counted after ten days of incubation at 37 °C in a humidified incubator containing air—5% CO$_2$ To determine BFU-E and CFU-GEMM, cells were cultured as described but with 30% FCS in the Iscove's medium–methylcellulose mixture plus 5×10^{-5} mol/L 2-mercaptoethanol, 0.9% deionized bovine serum albumin (BSA), 1 μ/mL human urinary erythropoietin, and 1% of a T cell line conditioned medium (MoCM).[20,21] Leptalb-7 (Bovuminar—30% solution, Armour Pharmaceutical Co, Tarrytown, NY) with added sodium bicarbonate (0.08%) provided the BSA used for aiding support of BFU-E and CFU-GEMM growth. Erythroid bursts (BFU-E) and CFU-GEMM were counted on day 14 of culture. The colonies were counted using an inverted

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microscope at 30× Representative colonies (10% to 20% of CFU-GM, myeloid clusters [possessing less than 50 cells per aggregate], BFU-E, and most CFU-GEMM) were picked, and cytocentrifuge preparations were made for Wright's-Giemsa and (for CFU-GM) chloroacetate and alpha naphthyl acetate esterase staining to assess their morphology.[22] All CFU-GEMM had a mixture of myeloid and erythroid cells, and some of these colonies also possessed megakaryocytes. To evaluate differentiation patterns of CFU-GM, three types of myeloid colonies were enumerated: "large cell," compact "small cell," and dispersed "small cell," which were found by Wright's-Giemsa and esterase staining to be monocyte-macrophages, blasts/immature granulocytes, and mixed immature/mature granulocyte-monocyte colonies, respectively. When assessed after ten days of incubation, the distribution of HPCM-stimulated normal marrow myeloid colony types was 5% ± 1%, 18% ± 2%, and 77% ± 3% (mean ± SE), respectively

Drugs, stimuli. 13-cis retinoic acid (retinoic acid), all-trans retinoic acid, retinol, and 1,25 dihydroxyvitamin D_3 (vitamin D) (kindly provided by Dr Milan Uskokovic, Hoffmann-LaRoche Inc, Nutley, NJ) were diluted in absolute ethanol (final concentrations ≤0.01%) and stored at −20 °C in containers gassed with nitrogen. Because these agents are light sensitive, the tubes and dishes containing them were shielded from light. CSF-GM for the in vitro studies was provided from HPCM.[20,23] Partially purified human urinary erythropoietin (1,140 U/mg protein) was obtained from the National Institutes of Health. The MoCM was kindly provided by Dr David Golde, UCLA Medical Center.[24]

Cytogenetics. Chromosome analyses were determined for native marrow cells and from cells incubated in vitro in clonogenic methylcellulose culture. Karyotypes from the prophase and prometaphase preparations of marrow cells thus obtained were analyzed by Giemsa banding after methotrexate/thymidine synchronization by Dr Fred Hecht and colleagues at the Southwest Biomedical Research Institute, Tempe, Ariz, as previously described.[25,26] A minimum of 15 chromosome spreads were analyzed per study. To obtain karyotypes of the cells cultured in vitro, the 1-mL methylcellulose-media gels containing the cells in the tissue culture plates were solubilized after ten days in culture by adding 6 mL of Iscove's medium plus 15% FCS (three 2-mL washes). The cell pellet obtained by centrifugation (400 g, five minutes) was resuspended, and 25 ng of Colcemid (GIBCO, Grand Island, NY) per 2 mL was added for two hours along with 15% HPCM, mixing gently every 30 minutes. The cells, obtained again by centrifugation, were resuspended in 2 mL of prewarmed 0.075 mol/L KCl for ten minutes at 37 °C. Cell fixation with 4 mL 3.1 methanol/glacial acetic acid was performed by slow mixing for ten minutes. The fixed cells were centrifuged, the cell pellet recovered, and 2 mL fixative was added. The cells were dispersed, incubated for ten minutes at room temperature, centrifuged, and resuspended in 1 mL of the fixative. From a height of about 15 cm, approximately 250 µL of cell suspension was added dropwise onto slides that had been presoaked in 70% ethanol and then flame dried.

Patients. Marrow specimens from 15 normal individuals (median age 59) and from 22 patients with MDS were studied. The 21 adult patients were elderly, median age 71 years old The MDS patients evaluated were separated into two groups. All patients were studied at initial clinical evaluation. Group I (ten patients) were subjects who received 13-cis retinoic acid therapy orally for eight weeks after in vitro marrow culture studies, five of whom were restudied within one to two weeks after discontinuing treatment. No patient had marrow culture studies performed during retinoic acid therapy. Group II (12 patients) were subjects who had the in vitro marrow culture studies performed but did not receive retinoic acid treatment (Table 1). Diagnostic criteria for the MDS (also termed smoldering myeloid leukemic states[1]), have previously been described and include refractory cytopenias for at least two months in patients with specifically abnormal marrow morphology. These syndromes have previously been categorized as hemopoietic dysplasia (preleukemia), smoldering or subacute myeloid leukemia (SML), and refractory anemia with excess of myeloblasts (RAEM).[1,2,27-32] Hemopoietic dysplasia has been defined as showing dysplastic maturation of marrow elements in at least two of the three major cell lines, including such qualitative changes as megaloblastoid erythropoiesis, ringed sideroblasts, defective myeloid differentiation, nucleocytoplasmic asynchrony, and dwarfed or abnormal megakaryocytes. SML has been defined by these characteristics and with 10% to 30% marrow myeloblasts, some degree of myeloid maturation, and an indolent clinical course. This categorization, rather than the French-American-British (FAB) reclassification proposal, has been used to describe MDS patients for reasons of historical usage and because of ambiguity of certain FAB formulations.[1] Eighteen of our patients had hemopoietic dysplasia and four had SML Initial clinical features of these individuals are given in Table 1. All of the patients were anemic, three were thrombocytopenic, four were neutropenic, and 11 pancytopenic. Ten study patients were treated with 13-cis retinoic acid (Accutane, kindly provided by Dr Loretta Itri, Hoffman-LaRoche) after informed consent was given, following guidelines established by the Stanford University Human Experimentation Committee. Treated patients received 13-cis retinoic acid orally, 2.5 to 4 mg/kg/d for eight weeks, and then were reevaluated with blood counts and marrow aspiration, and assessment of marrow morphology within one to two weeks after discontinuing the drug. In six of ten treated patients, marrow cytogenetics were also reevaluated at this time. Blood counts were obtained weekly throughout the course of treatment. Repeat in vitro marrow cell culture studies were also performed in five of these patients at this time. The clinical features of these patients and in vivo results of the therapeutic trial are being reported more extensively in a separate communication.[33] Serum retinoic acid levels achieved in individuals receiving 2.5 mg/kg 13-cis retinoic acid ranged between 0.29 and 0.91 µg/mL (1 to 3 × 10^{-6} mol/L) four hours after their ingesting the drug (kindly performed by Harris Laboratories, Lincoln, Neb). Statistical analyses were performed using Student's *t* test and chi-square test with Yates correction.

RESULTS

Clonogenic proliferative responses of normal marrow hemopoietic precursors to retinoids and vitamin D. After in vitro exposure of normal buoyant marrow mononuclear cells to 10^{-5} to 10^{-9} mol/L retinoic acid and 10^{-7} to 10^{-10} mol/L vitamin D, for the entire cell culture incubation period, maximal stimulation of CFU-GM was noted at 10^{-6} mol/L for retinoic acid (223% ± 15% increment over CSF-dependent basal values, $P < .01$) and at 10^{-9} mol/L vitamin D (153% ± 8% increment, $P < .05$) (Fig 1). In contrast, for erythropoietin-dependent BFU-E, a 30% to 50% decrement ($P < .05$) was caused by both agents (Fig 1). A similar decrement occurred when dimethyl sulfoxide (DMSO) rather than ethanol was used as diluent (data not shown). No change in colony formation occurred for CFU-GEMM.

To assess the specificity of the 13-cis retinoic acid effects, similar in vitro studies were performed with the related vitamin A analogues all-trans retinoic acid and retinol. Our studies with normal marrow cells showed no alteration of CFU-GM and BFU-E growth after retinol exposure in vitro at concentrations of 10^{-5} to 10^{-9} mol/L, n = 2 (Fig 2). In contrast, when using all-trans retinoic acid, moderate increments in CFU-GM growth (130% ± 12% of basal values,

Table 1. Clinical and Biological Features of Patients With Myelodysplastic Syndromes: Responses to 13-cis Retinoic Acid and 1,25 $(OH)_2$ Vitamin D_3

				Pretherapy			Pretherapy Marrow			
							In Vitro Findings			
Patient No	Diagnosis	Age/Sex	Time From Diagnosis (yr)	Platelet Count*	PMN Count*	Posttherapy Change PMN Count (% basal)	CFU-GM†	BFU-E†	CFU-GEMM†	Cytogenetics
Group I										
1‡	SML	39/M	0.8	31	12.5	145	9^{aB}	0^{dD}	0^{dD}	−16, two transl'ns
2‡	HD	65/M	0 2	211	1.4	114	6^{aA}	46^{eE}	0^{dD}	45x-y, 46xy^{m}
3‡	HD	64/M	7 0	40	2.0	170	31^{aA}	67^{e}	0^{d}	45x-y, 46xy^{m}
4‡	HD	76/M	0.4	39	1.3	173	63^{bB}	85^{eE}	0^{dD}	45x-y, 46xy^{m}
5‡	HD	90/M	6.0	121	0.4	700	63^{bB}	7^{eE}	0^{dD}	−18, 46xy^{m}
6	HD	70/M	1.0	49	4.9	122	197^{bB}	0^{dD}	0^{dD}	46xy iso(17q)
7	SML	73/M	0.5	82	1.1	136	104^{bB}	0^{dD}	0^{dD}	−9, −18, 46xy^{m}
8	HD	42/M	0.3	285	4.2	71	62^{c}	0^{d}	0^{d}	46xy
9‡	HD	76/M	0.4	27	0.2	67	43^{cC}	0^{d}	0^{d}	46 xy iso (17q),^{m} 47xy iso (17q)+19
10	SML	83/F	0.3	24	0.8	31	3^{c}	0^{d}	0^{d}	46xx,^{m} 45xx-5+ marker
Group II										
11	HD	80/M	2 0	78	0.1	—	10^{aA}	2	0	–
12	HD	65/M	2 0	145	0 3	—	43^{e}	109^{e}	1^{d}	46xy
13§	HD	71/M	0 3	·113	0.9	—	26^{aA}	0^{d}	0^{d}	46xy
14	HD	8/F	0.8	21	0.9	—	11^{b}	0^{d}	0^{d}	−7
15	HD	80/M	2 8	145	1 9	—	105^{c}	89^{e}	2^{d}	46xydel (20q), 46xy^{m}
16	HD	39/M	0 2	235	4 1	—	31^{c}	1^{d}	0^{d}	3 abn lines^{m}
17	HD	73/M	0.1	13	0 2	—	5^{cA}	0^{dD}	0^{dD}	46xy,45x-y^{m}
18	SML	72/F	0.4	40	0.3	—	10^{cC}	0^{d}	0^{d}	46xx
19§	HD	47/M	0.3	5	0.2	—	0^{d}	0	0	46xy
20	HD	74/M	5.0	467	3.3	—	0^{d}	—	—	46xy
21	HD	68/M	0.3	190	1.3	—	0^{d}	0^{d}	0^{d}	−5q, inv7
22	HD	58/M	2.0	410	1.3	—	0^{dD}	27^{eD}	0^{dD}	46xy

Growth patterns after in vitro exposure to retinoic acid (a through e) or 1, 25$(OH)_2$ vitamin D_3 (A through E)· a or A, increased CFU-GM and increased clusters; b or B, decreased CFU-GM; c or C, decreased CFU-GM and increased clusters; d or D, no change; e or E, decreased BFU-E growth. HD, hematopoietic dysplasia; SML, subacute myeloid leukemia; m, cytogenetic mosaicism.

*× 10^3 cells per mm^3.

†Per 10^5 marrow cells.

‡Also had in vitro marrow culture study or cytogenetics after treatment

§Post alkylator therapy MDS

$P < .05$) occurred, with a peak at 10^{-6} to 10^{-7} mol/L, and decrements (70% ± 7% of basal values at 10^{-6} mol/L, $P < .05$) in BFU-E growth occurred, n = 3 (Fig 2).

Clonogenic proliferative and differentiative responses of MDS marrow hemopoietic precursors to retinoids and vitamin D. Twenty-two patients with MDS were evaluated with in vitro marrow culture studies. In these 22 studies, buoyant marrow mononuclear cells from 18 patients showed formation of CFU-GM, nine formed BFU-E, and two formed CFU-GEMM (Table 1, Fig 3). Colony formation, when it occurred, generally had a lower plating efficiency than that of controls, and four patients formed no colonies. No augmentation of colony formation occurred after in vitro exposure to retinoic acid (unchanged colony formation, Fig 3) or vitamin D in those patients initially lacking myeloid, erythroid, or mixed colony formation ("non-colony form-

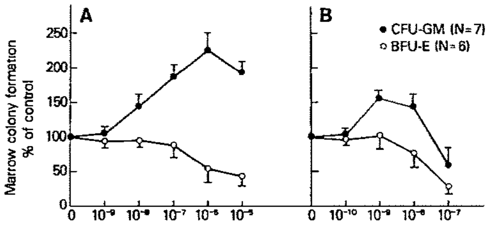

Fig 1. Proliferative responses of normal marrow myeloid (CFU-GM) and erythroid (BFU-E) precursors on in vitro exposure to (A) 13-cis retinoic acid; M, or (B) 1,25 dihydroxyvitamin D_3, M. Values represent means ± SE.

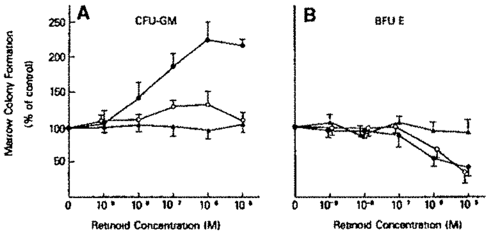

Fig 2. Proliferative responses of (A) normal marrow myeloid (CFU-GM) and (B) erythroid (BFU-E) precursors on in vitro exposure to various retinoid congeners: ●—●, 13-cis retinoic acid; ○—○, all-trans retinoic acid; and ▲—▲, retinol.

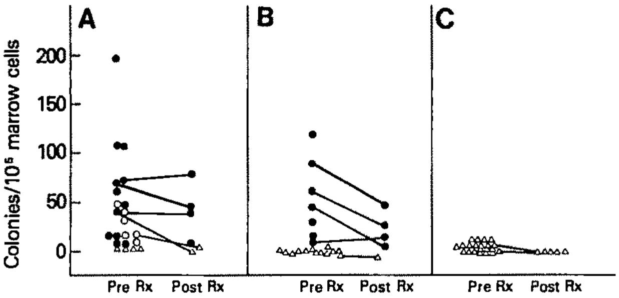

Fig 3. Marrow hemopoietic precursor [(A), CFU-GM; (B), BFU-E; (C), CFU-GEMM] colony formation in vitro from patients with myelodysplastic syndromes before and after in vivo treatment with 13-cis retinoic acid. Patterns of in vitro colony formation responses to in vitro exposure to 13-cis retinoic acid (10^{-6} mol/L) are also shown: ○, increased; ●, decreased; △, no change in colony formation.

ers") (Table 1). The same patients were simultaneously evaluated for growth of the three hemopoietic progenitor cells in the absence or presence of 10^{-6} mol/L retinoic acid (Fig 3). For the myeloid "colony formers," several patterns of in vitro response to the vitamins were noted, with more pronounced in vitro effects being seen after retinoic acid exposure than after vitamin D exposure (Fig 4). In the presence of retinoic acid, the number of CFU-GM was increased in six patients and decreased in 12 (13% to 100% change in colony formation, with the vast majority being >20% from baseline values; Fig 3). In 12 of these 18 patients, increased growth of clusters developed after in vitro exposure to retinoic acid. In vitro responses to retinoic acid of colonies and clusters together showed four growth patterns (a) increased CFU-GM and increased clusters (six patients); (b) decreased CFU-GM, no change in clusters (five patients); (c) decreased CFU-GM and increased clusters (six patients); (d) no change (five patients, four of whom initially formed no colonies). No association was found between initial colony-forming incidence and the pattern of response to retinoic acid. Further, no correlation was noted between initial clinical features, marrow morphology, cytogenetics, or in vitro responses to retinoic acid. When grown in the presence of vitamin D, an increase in CFU-GM incidence occurred in five patients, a decrease occurred in seven, and there was no change in one, compared with control values. Decreased BFU-E growth (22% to 96%; median, 33% of control) occurred in seven of the eight tested patients having erythroid growth ($P < .05$) after retinoic acid exposure but in only three of six patients after vitamin D exposure (NS). Except for two patients whose marrows had one to two mixed colonies per 10^5 cells plated, no CFU-GEMM were detected in marrow cells of the patients with or without vitamin exposure. Similar patterns of response of the marrow hemopoietic precursors from MDS patients occurred on in vitro exposure to retinoic acid and vitamin D (ie, concordance was found for 11 of 13 patients' myeloid growth patterns [CFU-GM, clusters], seven of eight patients' BFU-E, and all eight CFU-GEMM [Table 1, Fig 4]).

Effects on differentiation were assessed by noting whether an increase in the percentage of mature granulocyte or monocyte–macrophage type colonies occurred after exposure to these drugs. In the majority of patients, enhanced granulocyte–monocyte differentiation was noted after in vitro exposure to retinoic acid (in nine of 16 patients; 56%) or vitamin D (11 of 15 patients; 73%). Maximal changes in in vitro colony formation in MDS patients occurred at concentrations of retinoic acid (10^{-6} mol/L) and vitamin D (10^{-8} mol/L) that were similar to values obtained with marrow cells from normal individuals. No differences in differentiation potential were noted in cells with types (a), (b), or (c) growth patterns.

Biological effects of the in vivo therapeutic trial with 13-cis retinoic acid. Ten patients with MDS (group I) were treated with an eight-week course of oral retinoic acid, five of whom were restudied within one to two weeks after completion of therapy. No patients were studied during the time they were taking retinoic acid. Alterations of peripheral blood counts, marrow morphology, in vitro hemopoietic growth characteristics, and (see below) cytogenetic analyses were evaluated in these patients. After in vivo exposure to retinoic acid, these patients generally demonstrated no substantial changes from their initial colony-forming incidence or *patterns* of response to the drugs (Figs 3 and 5).

Correlations between in vivo hemopoietic responses to retinoic acid treatment and initial clonogenic growth patterns and in vitro responses to retinoic acid were evaluated (Table 1). Ten of the MDS patients (group I) studied with in

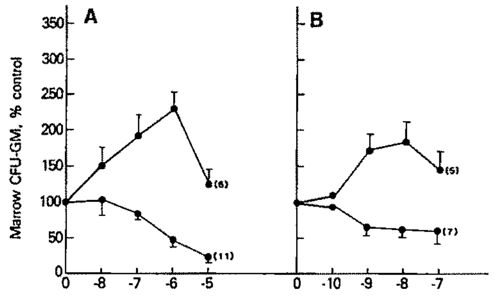

Fig 4. Proliferative responses of marrow myeloid precursors (CFU-GM) from myelodysplastic patients initially forming colonies who demonstrated increased (upper curve) or decreased (lower curve) colony formation upon in vitro exposure to (A) 13-cis retinoic acid, $\log_{10}$ M or (B) 1,25 dihydroxyvitamin D_3, $\log_{10}$ M.

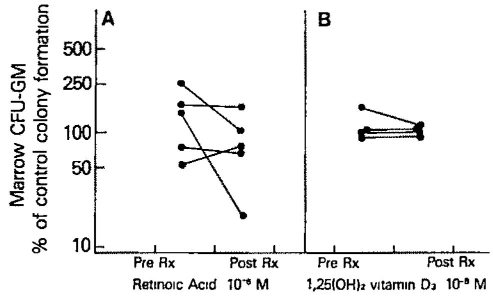

Fig 5. Proliferative responses of marrow myeloid precursors (CFU-GM incidence) from myelodysplastic patients upon in vitro exposure to (A) 13-cis retinoic acid 10^{-6} M and (B) 1,25 dihydroxyvitamin D_3 10^{-8} M and pre and post in vivo treatment with 13-cis retinoic acid.

vitro marrow clonogenic assays were treated with retinoic acid. Seven of the ten patients developed increments in peripheral blood neutrophil levels during therapy (114% to 700%; median, 145%) and three had decrements (31% to 71% of basal values; median, 67%). The improved neutrophil counts occurred by six to eight weeks of therapy and returned to basal levels within two to four weeks after cessation of therapy. There were essentially no changes in the patients' platelet counts, hemoglobin levels, or transfusion requirements during or immediately after the treatment course. No correlation was noted between in vivo responses to retinoic acid and the initial values for marrow CFU-GM, BFU-E, or CFU-GEMM incidence. All of these patients formed CFU-GM, and six had increased myeloid clusters (four patients whose neutrophils increased, two patients whose neutrophils decreased after therapy) in the absence of in vitro retinoic acid. After exposure of the marrow cells to 13-cis retinoic acid in vitro (10^{-6} mol/L), the myeloid growth patterns (a), (b), and (c) described earlier were noted. All seven patients with growth patterns (a) and (b), in response to in vitro retinoic acid exposure, subsequently developed increments in neutrophil counts after in vivo treatment, whereas the three patients with myeloid growth pattern (c) had decrements in their neutrophil counts (Table 1). These associations between in vivo responses and in vitro response patterns were statistically significantly different ($P < .05$). As BFU-E growth in treated patients either decreased (three patients) or (as with CFU-GEMM growth) was initially absent and did not alter (seven patients) after in vitro exposure to retinoic acid, correlations of BFU-E or CFU-GEMM responses with in vivo responses were not apparent. No correlations between marrow cytogenetic findings, clinical features (eg, presence or absence of neutropenia, other cytopenias, category of MDS), or in vivo responses to retinoic acid were noted in the group I patients.

Marrow cytogenetic analyses. Cytogenetic characteristics and marrow morphology of native marrow cells from normal and preleukemic patients before and after in vivo exposure to retinoic acid were determined. Twenty-one of the 22 patients had marrow cytogenetics assessed. Fourteen of the 21 patients (67%) had detectable chromosome abnormalities: four elderly males had cells that lacked the y chromosome in addition to normal cells, and ten had other abnormal karyotypes. Eight patients had chromosomal mosaicism with normal and abnormal cell lines, six had only abnormal chromosomes, and six had complex cytogenetic changes. In the group of ten patients receiving retinoic acid treatment (group I), one patient had a normal marrow karyotype, three were mosaics with loss of y chromosome, and the remainder had aneuploidy (Table 1). Shared chromosome abnormalities included partial deletion of chromosome 5 (patients 10 and 21), an isochromosome 17 (patients 6 and 9), and loss of the y chromosome in cells from four males (patients 2 through 4, 17). Six patients (1 through 5, 9) had posttreatment cytogenetics assessed, and with one exception (patient 5), no change was noted in these marrow morphological or chromosomal features after therapy. Patient 5, whose marrow chromosome pattern normalized after treatment, had morphological improvement in marrow granulocyte maturation and developed the most dramatic increase in blood neutrophil counts. In this patient, all of these parameters reverted to baseline values within one month after discontinuing treatment. No correlation was evident between marrow cytogenetics and the patients' clinical features, response to therapy, or in vitro hemopoietic growth patterns (Table 1).

Cytogenetic characteristics of both native marrow cells and marrow cells cultured in vitro from normal and MDS patients were determined. Cells stimulated by HPCM in methylcellulose clonogenic cultures were examined in the presence or absence of 10^{-6} mol/L retinoic acid exposure. Bone marrow cells cultured in vitro from two normal individuals (possessing normal marrow cell karyotypes) were analyzed and yielded normal karyotypes with or without in vitro retinoic acid exposure. Marrow cells from three MDS patients were examined in this fashion. One patient (12) had normal native marrow cell chromosomes and type (a) in vitro myeloid growth pattern responses to retinoic acid. This patient had normal metaphases demonstrated in cells obtained from methylcellulose gel cultures in the presence or absence of retinoic acid. Two MDS patients with either aneuploidy (patient 1) or a mixture of normal and aneuploid karyotypes (patient 15) were also evaluated. Patient 1's cells yielded chromosomes with the same abnormal karyotype as the native marrow in both the presence (wherein a 20-fold increase of clusters was generated) and the absence of in vitro exposure to retinoic acid. Both the normal and the abnormal karyotypes initially present in marrow cells from patient 15 were found after retinoic acid exposure in vitro, but only normal karyotypes were demonstrable in the absence of retinoic acid. Patient 1 also had persistence of his initial native marrow cytogenetics after in vivo treatment with retinoic acid.

DISCUSSION

These data describe clonogenic hemopoietic precursor proliferative and differentiative responses upon in vitro exposure of marrow cells from normal individuals and 22 patients with MDS to 13-cis retinoic acid and 1, 25 dihydroxyvitamin D_3. Confirming previous reports,[13,18] we showed augmentation of normal CFU-GM colony formation after in vitro exposure to 13-cis retinoic acid, all-trans retinoic acid, and vitamin D. The increases in normal marrow CFU-GM from vitamin D exposure were similar to those from retinoic acid but were less striking. These effects occurred at drug concentrations achievable by pharmacologic doses of retinoic acid administered in vivo (10^{-6} mol/L) but required doses higher than those attained (2×10^{-10} mol/L)[4] by treatment with 1,25 dihydroxyvitamin D_3. Substantial decrements in erythroid colony formation occurred after exposure to both drugs (again, requiring excessively high concentrations of vitamin D). The inhibitory effects on BFU-E growth were not attributable to the ethanol diluent, as control values with in vitro exposure to ethanol alone showed no such decrement, whereas use of another diluent (DMSO) in the presence of the vitamins demonstrated the inhibition. Prior studies have also shown no decrement in erythroid colony formation at the ethanol concentrations that we used ($\leq 0.01\%$).[34] As specificity controls, we demonstrated that retinol, a vitamin A analogue that is relatively inactive for hemopoietic growth-promoting functions,[13,15,16,35] caused no change in myeloid or erythroid colony formation in our subjects, whereas the active compound all-trans retinoic acid had effects similar to 13-cis retinoic acid (Fig 2). Significant decrements (median 33% of control) in BFU-E also occurred after in vitro retinoic acid exposure in the MDS patients who had erythroid growth (Table 1). This effect was not evident with vitamin D. The observed inhibitory effect of retinoic acid on BFU-E growth is at variance with one previously published study that found an increase in erythroid colony formation in the presence of retinoic acid.[15] We believe that the difference is at least partially attributable to the fact that the previous study examined circulating peripheral blood BFU-E, whereas we evaluated bone marrow erythroid precursors. Marrow and peripheral blood BFU-E have differing sensitivities to erythropoietin levels.[36] Concentration-dependent effects of marrow adherent cells on in vitro erythropoiesis have been demonstrated, with inhibitory effects on erythroid precursors occurring in the presence of high concentrations of these adherent monocytic cells or their culture supernatants.[37,38] Modulation of several functions of adherent cells (eg, macrophages) and subsets of T lymphocytes occurs after their exposure to retinoic acid.[39,40] Thus, functionally disparate accessory cell populations activated by retinoic acid or the BFU-E themselves, which are present within peripheral blood *v* bone marrow, may play a role in the contrasting results of our study and that previously reported. Further investigation of this issue is needed.

Low values for marrow CFU-GM and BFU-E frequency were found in the majority of marrow specimens from the MDS patients, as previously described.[41–47] The marrow cells from 18 of 22 MDS patients formed CFU-GM, nine formed BFU-E, but only two formed CFU-GEMM. The data, demonstrating marked diminution in CFU-GEMM colony formation in the MDS patients, indicate that these three precursors share the quantitative hemopoietic defects present in the disorder. The absence, rather than low numbers, of CFU-GEMM may reflect their relatively low incidence in marrow (and the limited quantity of cells plated). The addition of retinoic acid or vitamin D caused no augmentation of colony formation in those individuals initially lacking in vitro clonogenicity. The data indicate that the vitamins do not overcome the basic lesion(s) in in vitro proliferative potential of the nonclonogenic MDS hemopoietic precursors.

Increased differentiation of myeloid colonies to more mature granulocytes and monocytes was noted in the majority of MDS patients after in vitro exposure to retinoic acid (56%) or vitamin D (73% of patients). Other recent studies have also demonstrated enhanced monocyte–macrophage differentiation of colonies from normal and leukemic marrow after in vitro exposure to 1,25 dihydroxyvitamin D_3.[18,48] In the "colony formers," various patterns of response to retinoic acid and vitamin D in vitro were noted, with similar but more pronounced effects being seen with retinoic acid. Several patterns of myeloid growth after in vitro exposure to these agents were discerned: (a) increased CFU-GM and increased cluster formation, (b) decreased CFU-GM, (c) decreased CFU-GM and increased cluster formation, (d) no change. The varying responses of MDS cells may reflect our monitoring the growth of different proportions of normal *v* leukemic cells that were susceptible to proliferative or differentiative expression on exposure to these drugs. In addition, the presence of qualitative or quantitative differences in the accessory cells affected by these vitamins could have contributed to the diminished erythroid clonal growth patterns.

Biological effects of the relatively short (eight-week) trial of retinoic acid therapy on marrow cells were assessed in these MDS patients. After in vivo treatment of patients with retinoic acid, no clear alteration occurred in the in vitro colony-forming incidence or response to these agents for marrow CFU-GM, BFU-E, or CFU-GEMM. This finding was associated with the lack of alteration in marrow morphology or cytogenetics in virtually all of these patients. Correlations between in vivo responses to retinoic acid treatment and the responses of clonogenic myeloid cells to retinoic acid exposure in vitro were found: the seven MDS patients with patterns (a) and (b) after retinoic acid exposure in vitro subsequently developed increments in peripheral neutrophil counts after treatment, whereas neutrophil decrements developed in the three patients who had generated pattern (c) in vitro. No correlations were found between in vivo responses and values of initial marrow CFU-GM, BFU-E, or CFU-GEMM incidence in vitro or marrow cytogenetics. In a separate paper, we present more extensive data detailing the clinical features and in vivo responses of MDS patients to retinoic acid therapy.[33]

Nonrandom marrow cytogenetic abnormalities have been reported in a substantial proportion of patients with MDS[49–52] and were present initially in 14 of our 21 patients (67%) so evaluated. The recurring chromosomal abnormalities we found were partial deletion of the chromosome 5 long (q) arm, isochromosome 17, and loss of the y. These abnormalities are associated with MDS, and isochromosome 17 may be found in the blastic phase of chronic myelocytic leukemia.[1,25,49–52] Loss of the y chromosome is a common feature in elderly males and appears to be a reflection of aging.[53] These cytogenetic findings support the concept of the MDS as representing a clinically relatively indolent spectrum of clonal hemopathies. The clonal nature of these disorders and the qualitative cellular and biological abnormalities similar to those occurring in AML strongly suggest that the patients initially demonstrate critical neoplastic features. This group of disorders appears to represent coexisting normal and leukemic clones within marrow in which the host copes effectively with the leukemic clone or is populated by a leukemic clone with potential for greater in vivo differentiation.

To more clearly determine whether normal or abnormal clonal growth was altered by retinoic acid exposure, cytogenetic analyses were performed before and after retinoic acid therapy in six patients. Five of these individuals showed no alteration in their native marrow cell karyotypes. Three of these treated patients also had karyotypic analysis performed on cells present in their myeloid colonies grown in vitro in the presence and absence of retinoic acid. These in vitro and in vivo results demonstrated, with the one exception, the persistence or enhanced generation of the clones initially present in the patients' native marrow cells on retinoic acid exposure. The data are consistent with the hypothesis that retinoic acid increased the differentiation or proliferation of the endogenous marrow myeloid clones (normal and abnormal) in the MDS patients. The implications of enhanced abnormal (possibly leukemic/preleukemic) clonal proliferation in vitro in several of our patients need to be addressed. Clonal proliferation in the bioassay system that we used is accompanied by cell differentiation. Induced differentiation of leukemic cells has been shown to be associated with their decreased capacity for self-replication and leukemogenicity.$^{7-11,54}$ None of our treated patients had an increase in peripheral blood or marrow blast cells or underwent evolution to overt leukemia during the therapeutic trial. These findings provide no evidence for adverse hematologic effects of the retinoic acid exposure. However, further followup is needed to determine the long-term effects of this therapy on the patients' clinical outcomes. The temporary normalization of marrow cytogenetics in the one patient having the most striking improvement in neutrophils after in vivo retinoic acid therapy suggests treatment-induced improved growth potential of a karyotypically normal residual hemopoietic clone in this patient's marrow. The absence of correlation between marrow cytogenetic abnormalities and the in vitro or in vivo myeloid responses to retinoic acid suggest that factors distinct from detectable chromosome constitution regulate such an interplay (eg, retinoid binding proteins, effects of retinoids on accessory cells or on growth factors modulating hemopoietic precursors, alterations of cellular onc genes regulating cellular differentiation and proliferation).^{35} Such interactions between retinoids and growth factors have been demonstrated in other cell systems.^{35,55} Retinoic acid-induced differentiation is associated with altered expression of cellular oncogenes ^{56} Further evaluation is necessary and appears warranted to determine whether the natural history of MDS will be altered by more prolonged treatment of these patients with retinoic acid.

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Cytomegalovirus Infection After Bone Marrow Transplantation: An Association With Acute Graft-v-Host Disease

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Among 181 patients undergoing allogeneic bone marrow transplantation over a five-year period (1978 through 1982), cytomegalovirus (CMV) infection was a frequent and often lethal complication. Recipient pretransplant serology was the most important predictor of posttransplant CMV infection. CMV infection occured in 26/137 seronegative recipients and in 28/44 seropositive recipients ($P < .001$). Among patients who developed CMV infection, the time to infection was identical in seronegative and seropositive patients (median, 71 days post transplant). Bone marrow donor CMV serology did not significantly influence CMV infection rate. CMV infection was strongly associated with acute graft-*v*-host disease (AGVHD), occurring in 34/81 patients with AGVHD and 20/100 without GVHD ($P < .001$). AGVHD preceded CMV infection by 33.7 days (mean) in patients developing both complications. Patients who developed CMV infections had also received more cellular blood products post transplant. These data suggest that CMV infection may occur through reactivation of latent virus (in seropositive recipients) or through exogenous exposure, possibly through transfused blood products, but that duration of immunoincompetence may be more critical than route of exposure in timing of clinically evident CMV infection. Prophylaxis tailored to the likely infectious source and more effective GVHD prevention both may be critical in preventing CMV infection after bone marrow transplantation.

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BONE MARROW TRANSPLANTATION is increasingly successful in the treatment of a variety of hematologic and immunologic disorders. Although transplantation is effective in restoring hematopoiesis, the early complications of graft-*v*-host disease (GVHD) and infection (especially viral) limit the success of this treatment modality. Cytomegalovirus (CMV) infection has been documented in 40% to 50% of patients undergoing marrow transplantation, with adults developing CMV infection more frequently than pediatric patients.[1,2] In addition, interstitial pneumonitis, a severe and often fatal complication, is commonly associated with CMV infections.[1]

In bone marrow transplantation, immunosuppression is multifactorial. Immunoincompetence may result from the patient's primary disease, from the preparative regimen, from GVHD, or from treatment of GVHD. Several sources of CMV infection must also be considered. Immunosuppression may allow reactivation of latent virus in CMV seropositive patients. CMV might be transmitted with transplanted marrow from a seropositive donor. Finally, the multiple blood products that most patients receive in the early post-transplantation period may contain infective CMV particles. In an attempt to clarify the relative importance of these causes of immunosuppression and sources of infection, we analyzed the University of Minnesota experience with CMV in bone marrow transplantation over a five-year period.

METHODS AND MATERIALS

Patients. During a five-year period (1978 through 1982), 206 patients underwent allogeneic bone marrow transplantation at the University of Minnesota Hospitals Of these patients, 13 were eliminated from analysis because they were excreting CMV before transplantation and 12 patients were eliminated because of inadequate data regarding donor CMV serology. Thus 181 patients were retrospectively analyzed for their entire posttransplant course. Both adult and pediatric patients were included. Patients were grouped according to CMV serology before transplantation and by age (younger than 16 years old or 16 years or older). Forty-one recipients and 50 donors were judged seropositive on the basis of positive complement fixation titers, while three recipients and three donors were judged seropositive on the basis of positive immunofluorescence titers (with negative complement fixation). Blood, urine, and throat cultures for CMV were obtained and serum titers determined for each patient weekly during hospitalization and again at posttransplant day 100. Complement fixation titers were done on all samples, although not all negative complement fixation titers were retested by immunofluorescence. At day 100, both complement fixation and immunofluorescence titers were determined for all patients. Additional cultures were obtained as clinically indicated. Prospectively collected data regarding patient age, disease, conditioning regimen, GVHD, CMV infection, and survival were available from the University of Minnesota Bone Marrow Transplant Database Management under the direction of Dr Anne Goldman.

Definitions. Seropositivity for CMV was defined by a complement fixation titer of ≥1:8 or an immunofluorescence titer of ≥1:40. Seropositivity was interpreted as indicating prior exposure, not active infection. All CMV infections were diagnosed on the basis of positive CMV culture from any site, tissue biopsy showing typical CMV inclusions, or a fourfold increase in titers of antibody against CMV. Our methods for culturing CMV and performing humoral titers were as previously described.[3]

GVHD was diagnosed on the basis of clinical findings (rash, diarrhea, abdominal pain, hepatomegaly, jaundice, elevated liver enzymes), coupled with histologic changes of GVHD in biopsies of skin, liver, or gastrointestinal tract (stomach, duodenum, colon), using standard criteria.[4] Any patient with at least grade 1 GVDH was considered to have GVHD.

Blood product usage. We evaluated the number of cellular blood products administered to each patient included in the study. For each patient, red blood cell units, platelet donor units, and granulocyte donor units were enumerated. Pediatric patients

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received smaller packed red cell units (125 mL) than did adult patients (approximately 300 mL), but platelet units were the same size for pediatric and adult patients. Cellular blood products were enumerated for each patient during the first 35 days after transplantation. Because the usual onset of detectable CMV infection is at post-bone marrow transplant day 50 to 55,[1,2] it is likely that virus transmission or reactivation occurs during the early posttransplant period. In addition, CMV infection may increase transfusion requirements, and analysis of blood product requirements for the entire posttransplant course might not accurately reflect differences in blood product usage before CMV infection. We, therefore, evaluated blood product transfusion for each patient before the usual onset of CMV infection. (Complete transfusion data were not available for five patients. All five patients were seronegative, and only one developed CMV infection: a 14-year-old female, who died at posttransplant day 238 of relapsed acute lymphocytic. leukemia (AML). Of the remaining four, a 27-year-old male died at day +174 of relapsed chronic myelogenous leukemia (CML). The others remain alive without disease.)

Statistics. CMV incidence curves were estimated by the Kaplan-Meier product limit method similar to survival analysis, except that all patients were considered at risk for CMV until censoring at the time of death. The cumulative event proportions at one year were compared by t tests. Some factors that significantly affected CMV infection rates were also associated with one another (eg, age and GVHD). To study these associations, Cox multivariate regression analysis was carried out, using proportional hazards methods[5] for CMV incidence and for survival. For this purpose, we used the P2L program from BDMP Statistical Software, Inc (Los Angeles). GVHD occurs some time after transplant and was therefore treated as a time-dependent covariate in the Cox model.[6] Differences in blood transfusion amounts (comparing number of units received by patients developing CMV infection with the number of units received by patients remaining CMV-free) were analyzed using the Mann-Whitney rank sum test.

Autospy data. Autopsy reports were available for 50 patients included in this study group. Autopsy reports were reviewed for evidence of CMV infection and for distribution of CMV at autopsy. CMV was identified histologically by the presence of typical intracellular inclusions in tissue specimens.

RESULTS

CMV infections were documented in 29.8% of bone marrow transplant patients. CMV infection was diagnosed by positive cultures in 46 patients, compatible tissue morphology in two, and seroconversion in six patients. (Five of these patients were seronegative before transplant; the sixth patient had CMV titers of 1:16 by complement fixation and 1:20 by immunofluorescence before bone marrow transplantation that rose to 1:64 by immunofluorescence and 1:640 by complement fixation during the posttransplant course.) We evaluated several factors that might predict risk for development of CMV. The first of these, recipient and donor serology prior to bone marrow transplantation, are shown in Table 1. Among 137 seronegative patients, 26 (estimated infection rate, 22.8%) developed CMV infection, while 28/44 (estimated infection rate, 79.6%) of seropositive patients developed CMV infection during the posttransplant course ($P < .001$). The titer of CMV antibody present pretransplant, on the other hand, did not appear to influence infection rate. Among seropositive recipients, those with CMV complement fixation titers of 1:8 to 1:16 developed CMV infection in 15/21 cases. Recipients with complement fixation titers of 1:32 to 1:512 developed CMV infection in 14/23 cases. Thus recipient seropositivity (but not titer) was strikingly important in predicting CMV infection. Severity of infection was not apparently influenced by pretransplant serology, however. Twenty-three patients (12 seronegative and 11 seropositive) developed clinically apparent disease, while 31 patients had subclinical infections. Bone marrow donor serology, in contrast to recipient serology, had no significant influence on posttransplant CMV infection (32.7% *v* 47.6%, $t = 1.6, p = .12$) (Table 1).

We also analyzed the time to CMV infection for patients who actually became infected. The median time to diagnosis in patients developing CMV infection was 71 days post-bone marrow transplantation. Interestingly, neither recipient nor donor pretransplant serology appeared to influence the time to infection. Seropositive recipients and seronegative recipients had identical time curves (Kaplan-Meier) for acquisition of CMV infection, both with medians of 71 days. This analysis may be biased, since the 51 patients who died without CMV were not included in these particular calculations.

Patients developing CMV infections were somewhat older (mean age, 17.5 years) than patients remaining free of CMV (mean age, 13.9 years). Time to CMV infection was examined using the Cox regression method with age as a covariate. Increasing age was a significant predictor of time to CMV; each additional year of age was associated with a 1.032 proportional increase in the likelihood of CMV infection. The increased risk in adults appears to relate to the increasing incidence of seropositivity with increasing age. Seropositive recipients had a mean age of 21.1 years, whereas seronegative recipients had a mean age of 13.0 years. When seronegative patients were grouped by age, patients of less than 16 years of age (mean, 7.4 years) had an 18.8% incidence of CMV infection (16/85), while seronegative

Table 1. CMV Infection After Bone Marrow Transplantation: Relationship to Recipient/Donor Pretransplant CMV Serology

	Recipient Serology			
	Negative	Positive	Total	Estimated Incidence*
Donor Serology				
Negative	20/107	15/21	35/128	32.7% ± 4.7% SE
Positive	6/30	13/23	19/53	47.6% ± 8.3% SE
Total	26/137	28/44	54/181	
Estimated incidence†	22 8% ± 4.0% SE	79 6% ± 7.4% SE		

* *t* Test on cumulative proportion without event by donor serology is 1.56, $P = 12$

† *t* Test on cumulative proportion without event by recipient serology is 6.73, $P = .001$.

patients of 16 years or older (mean age, 22.2 years) had a nearly identical 19.2% (10/52) incidence of CMV infection. With Cox regression, age did not independently predict CMV infection when recipient serology and GVHD were included as covariates (Table 2).

The conditioning regimens used in bone marrow transplantation are a source of variation among patients. We evaluated the relationship between conditioning regimens and CMV infection rate. Seronegative patients receiving total body irradiation (chiefly for malignancies) had an 18.4% (19/103) incidence of CMV infection, while those receiving total lymphoid irradiation (chiefly for aplastic anemia) had a 23.3% (7/30) incidence of CMV infection ($\chi_c^2 = 0.11$, $P = .74$). Similarly, seropositive patients receiving total body irradiation had a 59.3% (16/27) incidence of CMV infection compared with 70.6% (12/17) for those receiving total lymphoid irradiation ($\chi_c^2 = 0.19$, $P = .66$). Four patients received chemotherapy without irradiation before transplantation, and none of these developed CMV infection.

The relationship between blood tranfusions and CMV infections in our patients is shown in Table 3. Patients who remained CMV-free received 33.3 units of blood (median), while those who developed CMV infection received more, with a median of 46.5 units of blood during the first 35 days after transplantation ($P = .08$). We also analyzed red blood cell and platelet transfusions for each patient. CMV-infected patients received significantly more red cell units (6.1 units, median) than did uninfected patients (5.0 units, median) ($Z = 1.96$, $P = .05$). CMV-infected patients also received more platelets (37.0 units) than did uninfected patients (25.5 units), although this difference was not statistically significant ($Z = 1.28$, $P = .12$ by Mann-Whitney). The products administered were not tested for CMV antibody. However, the overall incidence of CMV seropositivity in our blood donor population is 30%.

The association between acute GVHD (AGVHD) and CMV infection is shown in Table 4. Among patients with no AGVHD, 20% had posttransplant CMV infections, while 42% of those with AGVHD developed CMV infections. A Cox proportional hazards regression analysis was performed in which AGVHD was included as a time-dependent covariate. In this analysis of time to CMV, patients were included in the no AGVHD group until the time of diagnosis of AGVHD; at that point patients were switched to the AGVHD. Thus if CMV was evident before the diagnosis of AGVHD, the risk of CMV would be associated with no AGVHD; when AGVHD preceded CMV, the risk of CMV infection was associated with AGVHD. CMV infection was significantly more likely to occur among patients with AGVHD. The relative risk for those with AGVHD was 3.24 times that for those without AGVHD ($P < .001$). A similar regression analysis of time to CMV was conducted with chronic GVHD as a time-dependent covariate, but this analysis failed to show an association of CMV with chronic GVHD (relative risk of CMV = 1.63, $P = .40$). Among patients surviving longer than 100 days, chronic GVHD was diagnosed in 22/93 (23.7%) CMV-uninfected and 15/39 (40.5%) CMV-infected patients ($\chi_c^2 = 2.3$, $P = .13$).

To further clarify the relationship between AGVHD and CMV, we compared the time to CMV and time to AGVHD in 24 patients who developed both. Acute GVHD preceded diagnosis of CMV by a mean of 33.7 days in these patients ($t = 4.17$, $P < .001$). Because AGVHD preceded CMV infection in most patients, we next sought a relationship between treatment of AGVHD and CMV infection. Table 5 shows CMV infection incidence according to maximal GVHD therapy. Patients were treated with topical or moderate- or high-dose corticosteroids, depending on the distribution and severity of AGVHD. As can be seen, although AGVHD appeared to influence CMV infection rate, the dose or route of administration of GVHD therapy did not obviously affect CMV infection rate. Among seronegatives, the CMV infection rate was similar whether the patient received topical or moderate- or high-dose steroids ($\chi_c^2 = .44$, $P = .80$). Similarly, seropositive patients with AGVHD had nearly identical CMV infection rates regardless of GVHD therapy ($\chi_c^2 = .11$, $P = .74$).

Because recipient CMV serology, GVHD, age, and CMV infection were all interrelated factors, their association was further analyzed using Cox regression analysis. This method allowed us to test the association between rate of CMV infection and each of the factors while controlling for the effects of each of the remaining factors (Table 2). Both recipient serology and AGVHD were confirmed as significantly and independently related to CMV infection (both $P < .001$). Age, when we controlled for the effects of serology and AGVHD, did not contribute significantly to CMV risk ($P = .41$).

We evaluated the effect of CMV infection on survival. Of

Table 2. Cox Regression Analysis of Variables Relating to CMV Infections

Variable	Relative Risk	*P* Value
Age of recipient	0.99	.41
Recipient CMV seropositive	5.88	<.001
Acute graft-*v*-host disease*	3.45	<.001

*Treated as time-dependent covariate at time of onset

Table 3. Influence of Blood Transfusions on CMV Infections

Posttransplant CMV	Median Transfusions (Blood Product Donors)*	Range	Mean Rank (Mann-Whitney)
No CMV infection (n = 124)	33.3	0 to 330	84.1
CMV infected (n = 52)	46.5	6 to 391	98.8†

*Includes all red cell and platelet units transfused during the first 35 days after transplantation. No patient received granulocyte transfusions.

†$Z = 1.73$, $P = .08$.

Table 4. Incidence of CMV Infection According to Acute GVHD Status

Acute Graft-*v*-Host Disease	CMV Infection
No	20/100 (20.0%)
Yes	34/81 (42.0%)

Table 5. Incidence of CMV Infection According to Acute GVHD Treatment Regimen

Maximal AGVHD Therapy	CMV Infection Incidence	
	Seronegative Recipients	Seropositive Recipients
No GVHD	9/77	11/23
GVHD, no therapy	0/3	1/1
Topical steroid	5/14	3/5
Prednisone (60 mg/m^2/d)	7/27	5/6
Methylprednisolone (30 mg/kg/d)	5/16	8/9

patients developing CMV infection, 23/54 (43.0%) are alive one to five years after transplant, while 69/127 (54.3%) patients not developing CMV infection remain alive. Such data may not, however, accurately reflect the effect of CMV on survival. We therefore performed a multivariate analysis of the influence of various factors on survival. Factors included age (<16 *v* 16 or older), disease category (malignancy *v* other), high- *v* low-risk disease (see later), presence or absence of CMV infection, and presence or absence of GVHD. Patients were considered high risk if they were transplanted with acute nonlymphocytic leukemia (ANLL) in second or greater remission or in relapse; acute lymphocytic leukemia (ALL) in third or greater remission or in relapse; CML in accelerated phase, blast crisis, or post–blast crisis; neuroblastoma (three patients); or Burkitt's lymphoma transplanted in relapse (two patients). One patient with Fanconi's anemia was also included in the high-risk category. Patients were considered low risk if they were transplanted with ANLL in first remission; ALL in first or second remission; CML in chronic phase; or aplastic anemia. Using Cox regression analysis, we analyzed the independent effects of these factors on survival, using disease category, risk category, and age as baseline characteristics and including CMV and GVHD as time-dependent covariates, since they are not baseline characteristics. CMV infection was associated with decreased survival (relative risk 2.1, P = .005), as was high-risk disease (relative risk 1.72, P = .03). Age (relative risk 1.31), disease category (relative risk 1.13), and GVHD (relative risk 1.4) were not independent predictors of survival in this analysis. In a separate analysis of 101 leukemic patients, we compared the effects of diagnosis (ANLL *v* ALL *v* CML), age, risk, CMV infection, and GVHD. Diagnosis did not influence survival (relative risk 1.0), while CMV infection again showed a negative influence on survival (relative risk 2.4, P = .01), as did high-risk disease (relative risk 2.0, P = .025). This multivariate analysis clearly demonstrates that CMV infection was associated with an increased mortality and indicates that the overall risk of death among CMV-infected patients was 2.1 times that of those without CMV.

To further clarify the influence of CMV infection on mortality, we reviewed autopsy reports of 50 patients. Among 15 patients with autopsy evidence of CMV disease, 14 had interstitial pneumonitis. Only one patient had lung CMV without evidence of interstitial pneumonitis. There were an additional ten cases of interstitial pneumonitis not associated with CMV, so CMV was a candidate pathogen in 58.8% of cases of interstitial pneumonitis. The distribution of CMV in autopsy tissues included lung (15 cases), liver (six), stomach (five), colon (four), small bowel (three), esophagus (two), adrenal (two), skin (two), rectum (one), and kidney (one). Nineteen patients included in this autopsy series had antemortem culture or biopsy evidence of CMV infection, and 15 of those patients had CMV demonstrable at autopsy. Of 31 patients who did not have antemortem evidence of CMV infection, no patient had CMV at autopsy.

DISCUSSION

The data obtained in this study reveal or confirm several important points regarding CMV infection after bone marrow transplantation: CMV is an important source of posttransplant mortality, and seropositive recipients are not protected against severe infection. To the contrary, recipient (but not donor) seropositivity is an important predictor of posttransplant CMV infection. Red cell and platelet transfusions may be a source of CMV infection, possibly leading to infection in as many as 20% of patients, although the exact role of this infectious source was not clear in our studies. As previously observed,[1,7] there appears to be an important relationship between AGVHD and CMV infection, although we failed to confirm the relationship between CMV and chronic GVHD reported by Lönnquist et al.[8]

Seropositive patients developed CMV infections nearly three times as frequently as seronegative patients after bone marrow transplantation. This parallels the findings of Meyers et al[7] that recipient (but not donor) seropositivity is strongly associated with CMV pneumonitis. This implies that reactivation of latent virus may be an important source of clinical infection. The studies on the molecular epidemiology of CMV infections recently reported by Winston et al[9] provide excellent evidence that pretransplant CMV exposure may result in CMV disease after transplant. In those studies, four patients with asymptomatic CMV excretion before transplant developed CMV infection after transplant. In each case the post–bone marrow transplant isolate was genetically identical to the pretransplant isolate. Although reactivation of CMV has not been rigorously proven in humans, other herpesviruses may be reactivated after latent periods.[10] In addition, immunosuppression similar to that used in bone marrow transplantation has resulted in CMV reactivation in murine models.[11] Apparent transmission of CMV by granulocytes from seropositive blood donors points to latent viruses harbored by such donors that become infective when transfused into immunosuppressed hosts.[12,13] Thus it is likely that seropositive bone marrow recipients reactivate viruses that are latent in one or more body tissues. The relative contribution of other sources, such as blood transfusions, in seropositive individuals is unclear.

The bone marrow donor was not an obviously important source of CMV, since donor serology did not predict risk for development of CMV. This is in contrast to the situation in solid organ transplantation, where the transplanted kidney[14] or heart[15] from a positive donor may carry the virus, causing infection and disease in the recipient. Our data imply that the bone marrow from a seropositive donor is less infective than the kidney or the heart. The explanation for this difference is not clear. The infected cells may be quickly destroyed after bone marrow transplantation. Alternatively, during latency, the virus may not reside in the bone marrow in adequate numbers to be infective. It is also possible that the acquisition of CMV from other sources, such as blood products, may mask the contribution of the bone marrow donor, as suggested by the preliminary data of Bowden et al.[16]

Our finding that patients developing CMV infection received more transfusions before development of CMV suggests that red cell and platelet transfusions may transmit CMV in this population. Granulocyte transfusions have been clearly implicated in causing CMV infection after marrow transplant (Winston et al[12] and Hersman et al[13]). None of our patients received granulocyte transfusions. Yeager et al[17] reported that newborns receiving more than 50 pediatric blood units had a 24.4% incidence of CMV infection, but when infants received only screened CMV-negative blood products, 0/90 developed CMV infection. Preiksaitis et al,[15] in a much smaller study of cardiac transplant patients, found that using only CMV-seronegative blood products reduced the incidence of posttransplant CMV. The preliminary study of Bowden et al[16] also suggests that blood product screening is effective in preventing CMV in bone marrow transplant patients. These studies strengthen our impression that CMV is transmitted by red cell and platelet transfusions during the post-transplant course. The low incidence of CMV seropositivity in our blood donor population (30%) may in part explain our relatively low posttransplant CMV infection rate. A prospective study is now in progress in Minnesota to define the importance of blood transfusion in transmission of CMV infection after bone marrow transplantation.

The conditioning regimen used did not influence CMV infection rate. However, nearly all of our patients received radiation, either total body or total lymphoid. Among four patients who did not receive irradiation, no patient acquired CMV. Meyers et al[7] have emphasized that irradiation (even in low doses) predisposes to CMV pneumonitis. It is apparent from our patients that there is no difference between total body and total lymphoid irradiation as far as CMV risk. Our data complement those of Meyers in suggesting that irradiation (even in low doses) predisposes to CMV infection by enhancing immunosuppression rather than by damaging CMV target organs.

The nature of the relationship between GVHD and CMV infection is important. In our patients, GVHD usually preceded CMV infection. Although it is tempting to suggest that the immunosuppressive therapy used to treat GVHD allowed CMV infection, in fact, CMV infection was associated as much with AGVHD treated topically as with AGVHD treated with high-dose bolus methylprednisolone. These data suggest that the immunosuppression associated with AGVHD is more important than anti-GVHD immunosuppressive therapy in allowing reactivation of latent virus or dissemination of virus in newly infected patients. The alternative explanation that CMV infection predisposes to GVHD appears unlikely, since GVHD preceded CMV infection in most of our patients. CMV infections have been associated with a number of immunologic alterations, however, including reversal of the T4-T8 lymphocyte ratio,[18] alteration in cytotoxic T cell[19] and natural killer cell[20] activity, and enhanced expression of HLA-DR antigens.[21] In addition, increased rejection of renal allografts has been associated with the presence of CMV infection.[18,22] Thus CMV might enhance immune system reactivity, or it might alter the antigenicity of host tissues, thereby leading to an increased incidence of GVHD. If this is the case, prevention of CMV infection may decrease the incidence of GVHD. Indeed, Condie and O'Reilly observed a suggestive decrease in GVHD incidence when they prevented post-transplant CMV infection with prophylactic hyperimmune globulin.[23]

The sources of CMV infections appear to differ among bone marrow transplant patients. Seropositive patients may reactivate latent virus, while seronegative patients acquire CMV from exogenous exposure, possibly from blood transfusions. Immunosuppression caused by AGVHD appears to predispose to CMV infection. Our data suggest, however, that minimizing immunosuppressive treatment of GVHD may not decrease incidence of CMV infection, but that effective GVHD prevention may have an impact on CMV infection rates. Regardless of the source, CMV infections are frequently quite serious, with significant mortality and organ dysfunction. We are currently evaluating screened seronegative blood products for prevention of CMV infection in seronegative recipients at the University of Minnesota. Reactivation may be the primary source of CMV infections in seropositive patients; prophylaxis designed to kill or suppress reactivated virus may be necessary to prevent clinical CMV disease in seropositive recipients. Immunoglobulin with high anti-CMV titer or antivirals such as acyclovir, 9-[(2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine, or leukocyte interferon may prove useful when administered prophylactically to high-risk patients. The effectiveness of various forms of prophylaxis against CMV infection is currently under study, and we are hopeful that effective prophylaxis will improve results from bone marrow transplantation.

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CONCISE REPORT

Human Endothelial Cells Synthesize Protein S

By Daryl S. Fair, Richard A. Marlar, and Eugene G. Levin

Human umbilical vein endothelial cells were analyzed for the presence of prothrombin, factor VII, protein C, and protein S in culture supernatants and cell extracts using specific radioimmunoassays. Only protein S was detected. Conditioned medium from 24-hour cultures and cell lysates contained 21.7 ng/mL and 88.8 ng/10^7 cells of protein S, respectively. Intrinsic labeling and immunoprecipitation indicated that protein S was synthesized and secreted as a 75,000 molecular weight protein. Vitamin K, phorbol myristate acetate, and thrombin increased the production or specific activity (determined from activity/antigen ratios of 0.99 to 1.07, 0.93 to 1.04, and 0.90 to 1.04, respectively) of protein S. While untreated cells secreted a partially active protein S (activity/antigen = 0.40), warfarin greatly decreased the specific activity (<0.10) of this molecule, suggesting that endothelial cells contain the enzymes required for the carboxylation of selected glutamic acid residues. The production of protein S by these cells supports the hypothesis that cofactor production and expression by the endothelial cells may play a significant regulatory role in the initiation, propagation, and suppression of hemostasis and thrombosis.

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AMONG THE CELLULAR elements within the vasculature, platelets and endothelial cells have been implicated as principle components in the initiation, propagation, and suppression of hemostasis and thrombosis. One mechanism used by these cells is the synthesis or storage of specific proteins that amplify coagulation on their surfaces. Platelets store and release high molecular weight (mol wt) kininogen,[1,2] von Willebrand's factor[3] and factor V,[4,5] all of which are involved in clot formation or platelet aggregation. Recently, protein S has been found within platelets,[6] suggesting that these cells may also be responsible for limiting coagulation by the protein S-enhanced inactivation of factors Va and VIIIa by activated protein C.

Endothelial cells have also been shown to be a site for the initiation and propagation of coagulation.[7–10] In addition, these cells are unique in that they synthesize and express most of the cofactor activities associated with the activation and regulation of clotting. Among these components are tissue factor,[7,8] von Willebrand's factor,[11] factor VIII,[12] factor V,[13] high mol wt kininogen,[14] and thrombomodulin.[15]

We hypothesized that one of the general biological functions of endothelial cells is to control hemostasis and thrombosis by the production and expression of the cofactors that amplify these activities. Because protein S is a cofactor for activated protein C,[16] we tested our supposition by investigating the synthesis of this molecule by these cells. In this report, we demonstrate that human umbilical vein endothelial cells synthesize and secrete protein S, which can be modulated by warfarin and vitamin K. Thus this finding supports our hypothesis that all of the known cofactors required for the initiation, propagation, and suppression of coagulation are produced by the endothelial cells.

MATERIALS AND METHODS

Endothelial cells were isolated from human umbilical cord veins as previously described[17] and grown to confluence in T-75 flasks precoated with 2 mg/mL calf skin gelatin (Eastman Kodak Co, Rochester, NY). Cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum (Reheis Co, Phoenix), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO, Grand Island, NY). Conditioned medium containing protein S was collected in RPMI 1640 in the absence of serum for 24 hours. In selected experiments, the cells were treated with phorbol myristate acetate (PMA, 400 ng/mL; Sigma Chemical Co, St Louis), alpha-thrombin (1 U/mL, a gift from Dr John Fenton, New York Department of Health, Albany), vitamin K_1 (AquaMEPHYTON; Merck, Sharp & Dohme, West Point, PA; 10 μg/mL) or warfarin [3(α-acetonyl benzyl)-4-hydroxycoumarin; Sigma; 1 μg/mL] for 16 to 24 hours in RPMI 1640 containing 0.1% bovine serum albumin.

Protein S was purified from plasma by the method of Dahlback [18] Measurement of protein S antigen was by either a double antibody competitive equilibrium radioimmunoassay[19] on undiluted samples or a Laurell electroimmunoassay with Coomassie staining[20] on culture supernatants concentrated 20-fold and dialyzed by ultrafiltration using an Amicon device (Amicon Corp, Danvers, Mass). Recovery of protein S was 80% to 85% as estimated, using iodinated protein S added before concentration. Activity was quantitated by a modification of the method of Comp et al,[21] where Russell's viper venom (1:4,000 dilution; Sigma) was used in place of pure factor Xa. The sensitivity of the assay was to 3% of normal human plasma with a precision of 7% to 10% within and between assays. Dilutions of normal plasma and purified protein S were used to construct standard curves for activity and antigen measurements. The values were expressed as a percentage of a normal plasma pool.

Cultures of confluent endothelial cells were washed with 4 mL of methionine-free minimal essential medium (Irvine Scientific, Santa Ana, Calif) and incubated in 4 mL of the same medium supplemented with 5 μg/mL vitamin K and 150 μCi/mL ^{35}S-methionine (Amersham Corp, Arlington Heights, Ill) for six hours at 37 °C.

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After incubation, the supernatant was clarified by centrifugation at 100,000 g for 45 minutes at 4 °C, and the cell lysates were collected in 2 mL of 0.1% Triton X-100 (Eastman Kodak) containing 10 mmol/L benzamidine (Sigma), 1 mmol/L ethylamine diamine tetraacetic acid (EDTA), and 100 U/mL Trasylol (Bayer, Leverkusen, West Germany). Bovine serum albumin (5 mg/mL) was added to both the supernatant and cell lysate and immunoprecipitated using rabbit anti-protein S and *Staphylococcus aureus* exactly as described previously.[22] The immune complexes were solubilized into 2% sodium dodecyl sulfate (SDS) and electrophoresed on SDS-9% polyacrylamide slab gels containing ^{14}C-protein standards (New England Nuclear Corp, Boston) in the presence of 1% 2-mercaptoethanol.[23] Gels were fixed in 10% acetic acid, 25% methanol for one hour, washed extensively with water, incubated with 1 mol/L sodium salicylate for 30 minutes, dried, and subjected to autoradiography.

RESULTS

The production of protein S and other vitamin K-dependent proteins by human endothelial cells was analyzed by using specific radioimmunoassays for prothrombin, factor VII, protein C, and protein S. Only protein S was detected in significant quantities, with 88.8 ± 17.3 ng in the cell lysates from about 1×10^7 cells. Conditioned medium from 24-hour cultures contained 21.7 ± 3.3 ng protein S per milliliter, but none of the other molecules. Thus, of the four vitamin K-dependent proteins tested, only the molecule with cofactor activity was observed within the limits of this analysis.

To demonstrate that the endothelial cell synthesized protein S, we cultured the cells for six hours in the presence of radioactive methionine and immunoprecipitated both the supernatants and cell extracts with antibodies to protein S. We analyzed these cells for the production of von Willebrand's factor as control. Figure 1 is a representative example of five experiments in which the supernatant from endothelial cells was immunoprecipitated with rabbit anti-protein S in the absence and presence of 50 μg of exogenous purified protein S. A unique band of 75,300 ± 2,100 mol wt was precipitated with the antibody but was absent when unlabeled protein S was added to the supernatant before immunoprecipitation. The migration of this band was identical to iodinated native protein S (data not shown). Protein S represented about 0.1% of the total secreted protein relative to total trichloroacetic acid precipitable counts. Visualization of protein S in cell lysates was obscured by the coprecipitation of a nonspecific protein with a similar mol wt. When antibodies to von Willebrand's factor were used, the characteristic polypeptide chains of >220,000 daltons were observed (data not shown). Thus human endothelial cells synthesize and secrete protein S.

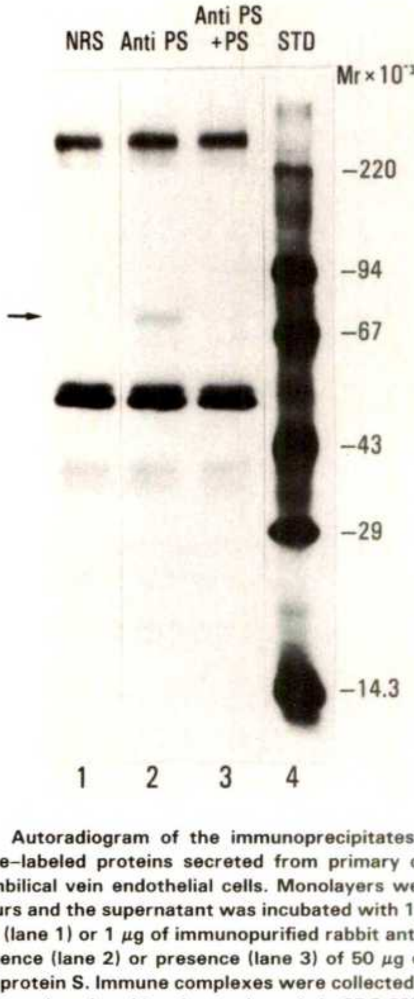

Fig 1. Autoradiogram of the immunoprecipitates from ^{35}S-methionine–labeled proteins secreted from primary cultures of human umbilical vein endothelial cells. Monolayers were labeled for six hours and the supernatant was incubated with 1 μg normal rabbit IgG (lane 1) or 1 μg of immunopurified rabbit anti-protein S in the absence (lane 2) or presence (lane 3) of 50 μg of purified, unlabeled protein S. Immune complexes were collected by binding to *S aureus* and analyzed by electrophoresis in SDS-9% polyacrylamide slab gels in the presence of 1% 2-mercaptoethanol. The migration of the labeled protein S-specific band is indicated by the arrow. Standards (lane 4) were myosin (220,000), phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), and lysozyme (14,300).

The production of protein S from these cells implies that they may contain the carboxylase enzyme system required for the characteristic posttranslational modification of selected amino-terminal glutamic acid residues.[24] To investigate this possibility, we tested the effect of warfarin, vitamin K, thrombin, and PMA on the ability of these cells to produce functional molecules. Confluent cells were cultured for 24 hours in the absence or presence of these agents, and the secreted protein S was assayed for activity and antigen using both normal plasma and purified protein S for standard curves (Table 1). Neither the medium or medium containing PMA in the absence of cells had protein S activity or antigen. Cells cultured in medium only released a partially active protein S as judged by the activity/antigen ratios of 0.35 to 0.40. Addition of thrombin did not increase the production of protein S significantly, but the functional capacity of the molecule was greater, as indicated by the increase in the activity/antigen ratio of 0.90 to 1.04. Both PMA and vitamin K enhanced the accumulation of protein S, which had specific activities of 0.93 to 1.04 and 0.99 to 1.07, respectively. Warfarin decreased the secretion of protein S slightly, but demonstration of a functional molecule was below the limits of detection in our bioassay. These trends were observed in three separate experiments. Within the sensitivity of our assay methods, the production of functionally active protein S can be increased by vitamin K, PMA, and thrombin and is significantly decreased by warfarin, a known inhibitor of the carboxylation reaction.

Table 1. Modulation of Protein S Activity and Antigen Secreted From Human Endothelial Cells

Culture Supernatant	Protein S					
	Activity		Antigen		Activity/Antigen	
	NHP	PS	NHP	PS	NHP	PS
RPMI	<3	<1	<3	<1	—	—
RPMI + PMA	<3	—	<3	—	—	—
Control CM	14	3.3	35	9.5	0.40	0.35
PMA CM	51	22.2	49	23.8	1.04	0.93
Thrombin CM	35	15.0	39	14.4	0.90	1.04
Vitamin K CM	48	20.0	45	20.2	1.07	0.99
Warfarin CM	<3	<1	29	12.1	<0.10	<0.10

Confluent cultures of human umbilical vein endothelial cells were incubated in the presence of phorbol myristate acetate (PMA, 400 ng/mL), alpha-thrombin (1 U/mL), vitamin K (10 μg/mL), or warfarin (1 μg/mL) diluted in serum-free medium containing 0.1% bovine serum albumin. Condition medium (CM) from 24-hour cultures were collected and concentrated 20-fold before analysis for activity and antigen. Activity and antigen values are the means for two experiments and are expressed as percentages of pooled normal human plasma assuming protein S concentration in plasma was 29 μg/mL. Standard curves for activity and antigen measurements were based on dilutions of normal human plasma (NHP) or purified protein S (PS).

DISCUSSION

Synthesis of vitamin K-dependent blood coagulation proteins was once thought to be restricted to hepatocytes. Indeed, our lab has demonstrated that a human hepatoma cell line can produce most of these molecules, including protein S, which represent their plasma protein counterparts immunochemically, structurally, and functionally.[22,25] Recently, the human monocyte and macrophage have been demonstrated to synthesize factor VII.[26,27] In this report, we have shown that the endothelial cell has the capacity to synthesize and secrete protein S, which has a similar mol wt as the plasma form,[19,28] and that its synthesis and activity can be affected by known pharmacologic modulators of vitamin K-dependent proteins. The finding that vitamin K increases and warfarin decreases the synthesis and specific activity of protein S suggests that they contain the enzymes required for gamma carboxylation. Thus at least two additional cell types have the capacity to produce functionally active members of this protein family but appear to be limited or restricted to the synthesis of a particular component.

The increase of protein S levels after thrombin addition would be physiologically consistent with the role of thrombin in protein C activation.[15] It can be envisioned that the increase in activated protein C mediated by thrombin–thrombomodulin would necessitate an increase in protein S levels to attain maximum protein C activity. The simultaneous occurrence of increased protein S-activated protein C levels could be coordinated by one endothelial cell. Studies determining the course of protein S release and the role thrombomodulin might play in protein S release will help to clarify the link between activation of protein C and protein S release.

The synthesis of protein S by endothelial cells may be biologically significant. Patients undergoing warfarin therapy show a decrease in vitamin K-dependent proteins, including protein S, of about 50%[19]; however, individuals with liver disease show a discordant reduction in protein S (~25% decrease) relative to protein C (~50% reduction), suggesting that there is another site of synthesis for protein S.[29] Our studies indicate the endothelium as a likely source of this molecule and would explain this apparent discrepancy. Recently, Stern et al[30] have shown that the endothelium can serve as a functional surface for the assembly of the activated protein C–protein S complex. In addition, they have evidence for the internalization of protein S by these cells in the absence of activated protein C, but protein S remains surface associated in its presence.[31] Our data suggest that the endothelial cell constitutively synthesizes and secretes protein S, and internalizes this molecule in a dynamic manner. Once low levels of activated protein C are formed by thrombin–thrombomodulin on the endothelial surface,[15] it may be in proximity to protein S (receptor), resulting in the formation of an active, stable inactivator complex for factors VIIIa and Va. The demonstration that this cofactor of activated protein C activity is produced by this cell supports our original hypothesis that cofactor production and expression by the endothelial cells may play a significant regulatory role in the initiation, propagation, and suppression of hemostasis and thrombosis.

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CONCISE REPORT

Hyperacute Graft-v-Host Disease in Patients Not Given Immunosuppression After Allogeneic Marrow Transplantation

By K.M. Sullivan, H.J. Deeg, J. Sanders, A. Klosterman, D. Amos, H. Shulman, G. Sale, P. Martin, R. Witherspoon, F. Appelbaum, K. Doney, P. Stewart, J. Meyers, G.B. McDonald, P. Weiden, A. Fefer, C.D. Buckner, R. Storb, and E.D. Thomas

Sixteen patients with leukemia in relapse or second to third remission, 5 to 27 years old (median, 17), were given cyclophosphamide (60 mg/kg × 2) and total body irradiation (2.25 Gy for each of seven days) followed by unmodified marrow grafts from HLA-identical siblings. Patients did not receive posttransplant immunosuppression and were followed a median of nine months (range, 5–17). Prompt engraftment was sustained in 12 patients with a median time of 16 days (range, 10 to 63) to achieve 500 neutrophils/mm^3. One patient failed to engraft, one had delayed engraftment, and two had late poor graft function. All 15 with engraftment developed moderate to life-threatening graft-*v*-host disease (GVHD, eight grade II and seven grade III-IV). This syndrome was hyperacute (median onset eight days [range, 7 to 29] posttransplant) and manifest by severe skin disease (14 patients at stage 3 and one at stage 4), fever (ten patients), and liver (four patients, stage 3-4) or gut (four patients, stage 3-4) involvement. Serial tissue biopsies confirmed acute GVHD in 13 of 15 patients. Ten were treated with antithymocyte globulin and cyclosporine (four survive), and four with corticosteroids (two survive). Actuarial survival to 17 months was 37%. Causes of death included interstitial pneumonia (four), infection (three), graft failure (one), venocclusive disease (one), and relapse of leukemia (one). Age-matched controls receiving standard methotrexate after transplant had comparable relapse-free survival but only a 25% incidence of grade II-IV acute GVHD ($P < .0001$). We conclude that deleting posttransplant immunosuppression is associated with frequent and severe hyperacute GVHD, infectious complications, and occasional poor graft function.

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ACUTE graft-*v*-host disease (GVHD) and associated infection and interstitial pneumonia (IP) remain major determinants of morbidity and mortality following HLA-identical marrow transplantation. Demonstration that GVHD could be averted or modified in animals if immunosuppressive agents such as methotrexate (MTX), cyclophosphamide (CY), or cyclosporine (CsA) were given after transplant led to direct clinical application.[1-4] However, immunosuppressive agents may contribute to delayed engraftment, IP, or infection and might not always be needed in humans. Moreover, previous clinical trials comparing different prophylaxis regimens could be invalidated if compared agents were equally ineffective in preventing GVHD.

The first study deleting immunosuppression after allogeneic transplant reported no difference in incidence or severity of acute GVHD in patients who did or did not receive MTX.[5] However, MTX recipients appeared to have more GVHD than expected for their age. Based upon these findings, it seemed justified to study young patients not given immunosuppression after marrow grafting. We report the incidence and severity of GVHD, IP, and infection in this trial and compare results with age-matched historic controls who differed only by receiving standard MTX.

MATERIALS AND METHODS

From March 1984 to March 1985, 16 study patients <30 years of age did not receive posttransplant immunosuppression. Protocols and consent forms were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. Attending physicians fully outlined the advantages and disadvantages of the procedure. Controls were derived from a randomized trial of patients less than age 30 conducted from Dec 1980 to July 1984 comparing standard MTX to other MTX-based regimens.[6] A total of 44 patients in that trial received standard intravenous (IV) MTX, 15 mg/m^2 day 1 and 10 mg/m^2 days 3, 6, and 11, then weekly to day 102.[2]

Patient characteristics are shown in Table 1. Patients in both groups received CY (60 mg/kg × 2), followed by 15.75 Gy total body irradiation (TBI, 2.25 Gy daily × 7). Both groups were comparable for factors influencing GVHD[7] and received unmodified marrow from HLA-identical siblings.[2] Engraftment was confirmed by peripheral counts, marrow aspirates, and cytogenetic markers. The assessment, grading, and treatment of acute and chronic GVHD have been described.[2,8] Grade II-IV acute GVHD was treated with prednisolone (2 mg/kg/d) or antithymocyte globulin (ATG, 15 mg/kg × 3) and CsA (3 mg/kg/d IV or 12.5 mg/kg/d orally).[9]

RESULTS

Engraftment. Engraftment was more rapid in those not receiving MTX (Table 2). Only one patient failed to engraft: due to electrocardiographic abnormalities, she received only 60 mg/kg CY followed by 15.75 Gy TBI. Three others not receiving immunosuppression had graft problems: one had delayed myeloid and platelet recovery despite early GVHD

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Table 1. Patient Characteristics

Factor	Posttransplant Regimen	
	No Immunosuppression	Standard MTX
No. of patients	16	44
Patient age		
Median (range) in years	17 (5–27)	19 (1–29)
No <10 yr old	4 (25%)	10 (22%)
Patient sex (M/F)	9/7	27/17
Patient–donor sex mismatch	9 (56%)	24 (55%)
Diagnosis		
Refractory ANL/ALL	1/0	1/0
Relapse ANL/ALL	3/1	11/12
2nd-3rd remission ANL/ALL	2/7	0/6
CML acceleration/blast crisis	1/1	3/9
Lymphoma	0	2
Median (range) no. of marrow cells infused ($\times 10^{-8}$/kg)	2.6 (1.2–5.1)	2.2 (0.9–11.5)
Median (range) follow-up in months	9 (5–17)	32 (14–56)

MTX, methotrexate; ANL, acute nonlymphoblastic leukemia; ALL, acute lymphoblastic leukemia; and CML, chronic myelogenous leukemia.

(63 days to 500 neutrophils/mm^3) and two had late graft failure after acute GVHD development.

Hyperacute GVHD. All 15 study patients who engrafted developed grade II-IV acute GVHD (Table 2). The onset was hyperacute (median, day 8) and associated with stage 3-4 skin disease (generalized erythroderma or desquamation) in all 15 patients. Fever ≥40 °C accompanied the onset in ten patients. Hepatic and enteric GVHD developed in most patients but was less severe than the dermal component: four had stage 3-4 liver disease (bilirubin ≥6 mg/dL) and four had stage 3-4 gut disease (diarrhea ≥1,500 mL/d, severe pain or ileus). Although difficult to distinguish between early GVHD and the histologic effects of chemoradiotherapy,[10] serial skin, liver, or gut biopsies confirmed GVHD in 13 patients. Treatment of hyperacute GVHD was started eight to 22 days (median, day 10) after transplant; ten patients received ATG and CsA (four survive) and four received corticosteroids (two survive). Six had sustained response, four had no benefit, and four had response followed by GVHD flare on tapering therapy.

Table 2. Posttransplant Regimen and Course

Factor	Posttransplant Regimen	
	No Immunosuppression	Standard MTX
No. of engrafted patients	15	44
Median (range) day to 500 neutrophils/mm^3	16 (10–63)	24 (17–35)
Median (range) day to last platelet transfusion*	45 (12–131)	32 (16–92)
Overall grade acute GVHD		
0 (None)	0	22
I (Mild)	0	12
II (Moderate)	8	1
III-IV (Severe, life-threatening)	7	9
Onset grade I-IV acute GVHD		
Median (range) day of onset	8 (7–29)	25 (11–65)
No. with onset ≤ day 14	11 (73%)	4 (18%)
No with stage 3-4 GVHD/total with stage 1-4 GVHD of:		
Skin	15/15	6/22
Liver	4/7	6/8
Gut	4/10	3/6
No. with interstitial pneumonia	4 (27%)	9 (20%)
No. with relapse of leukemia	1 (7%)	13 (30%)
No. with chronic GVHD/total no. surviving ≥180 days	2/6 (33%)	9/27 (33%)

MTX, methotrexate.

*Among patients surviving ≥85 days. Day 0 is the day of transplant.

Fig 1 depicts the incidence and time to onset of GVHD. Patients receiving MTX had a 25% incidence of grade II-IV acute GVHD compared with 100% incidence in those not given immunosuppression ($P < .0001$).

Infection and survival. Six of 16 study patients developed bacteremia, two cytomegalovirus (CMV) viremia, one aspergillus pneumonia, one toxoplasmosis, and one fulminant hepatic varicella. Four had IP (two pneumocystitis, one CMV, and one idiopathic) and three had hepatic venocclusive disease (VOD). Chronic GVHD developed in two of six surviving ≥180 days. Four study patients died of IP, three of infection, and one each of graft failure, VOD, and relapse. Fig 2 presents actuarial survival. Although relapse-free survival was comparable in study patients and controls, death from nonrelapse causes was increased in the no immunosuppression group ($P = .067$).

DISCUSSION

Based upon controlled studies in experimental animals, patients have routinely received posttransplant immunosuppression to prevent or ameliorate GVHD. The efficacy and toxicity of prophylaxis are incompletely understood since few patients have not received immunosuppression after transplant. We selected young patients with advanced leukemia for this pilot study since there might be either no increase in GVHD if prophylaxis were deleted,[5] or a possible antileukemic benefit if GVHD were augmented.[11] If these pilot data suggested no additional hazard, we proposed a subsequent randomized trial comparing MTX and placebo.

We found, however, that deleting posttransplant immunosuppression was associated with development of grade II-IV

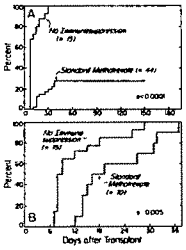

Fig 1. Kaplan-Meier estimates (generalized Wilcoxon test statistic). (A) Probability of developing grade II-IV acute GVHD. (B) Time to onset of grade II-IV acute GVHD. One patient in the no immunosuppression group failed to engraft and is excluded from analysis.

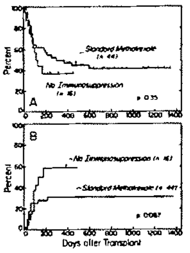

Fig 2. Kaplan-Meier estimates (generalized Savage test statistic). (A) Probability of relapse-free survival. Vertical marks represent patients surviving in remission as of Aug 1, 1985. The actuarial estimate of relapse of leukemia was 11% in the no immunosuppression group and 40% in the standard methotrexate group ($P = .27$). (B) Probability of death from nonrelapse (ie, transplant-related) causes. Patients are censored from analysis at the time of relapse.

GVHD in all 15 patients who achieved engraftment. This hyperacute syndrome included severe skin disease and fever beginning seven to 14 days after transplant. Features were sufficiently severe to require prompt treatment. Although response was often dramatic and perhaps blunted development of severe liver and gut involvement, GVHD flare was observed when therapy was tapered in four of ten who initially responded. Survival was poor with most deaths resulting from IP or infection. In contrast to others,[5,12] we noted an equal incidence of IP in those who did and did not receive MTX. Any lessening of pulmonary toxicity in patients not given MTX may be offset by increased GVHD and associated immunodeficiency.[13]

Age-matched controls given the same CY and TBI preparation and standard MTX after transplant had a 25% incidence of grade II-IV acute GVHD.[6] This agrees with other reports of infrequent GVHD in young patients receiving MTX[14,15] but differs from the study by Lazarus et al where 24 of 34 patients (71%) (median age, 12 years) given standard MTX developed grade II-IV acute GVHD.[5] The high incidence in the MTX controls may explain why that study found no increase in GVHD in 21 patients not given MTX.

The development of graft problems in four of our 16 study patients was not expected. One patient given a reduced dose of CY failed to engraft despite 15.75 Gy TBI. Three developed severe early GVHD with either delayed myeloid recovery or poor late graft function. We have observed such "lymphoid" grafts in GVHD patients who required marrow reinfusion to correct poor myeloid and platelet recovery.[16] Alternatively, deleting posttransplant immunosuppression could encumber engraftment. Studies in dogs suggested that MTX facilitated engraftment but the mechanism of the effect was never clear.[17]

The present data would refute the theory that previous trials of single agent prophylaxis were ineffective in preventing GVHD. Moreover, both animal[18] and clinical studies[14] have reported a reduction in the incidence of GVHD when multiagent immunosuppression was given after transplant. A recent trial comparing CsA to a combination of CsA and MTX showed both a reduction in GVHD and an increase in survival in patients receiving combination prophylaxis.[19] Such studies illustrate the interrelationship of graft function, GVHD, immunosuppression, IP, infection, and survival following allogeneic transplant. We conclude from the present study that deleting posttransplant immunosuppression is associated with frequent and severe hyperacute GVHD. Elimination of MTX does not appear to prevent IP or infectious complications. Occasional poor graft function may result from deletion of posttransplant immunosuppression, development of GVHD, or other factors.

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CONCISE REPORT

Cultured Human Endothelial Cells Synthesize a Plasma Membrane Protein Complex Immunologically Related to the Platelet Glycoprotein IIb/IIIa Complex

By O.C. Leeksma, J. Zandbergen-Spaargaren, J.C. Giltay, and J.A van Mourik

We have previously demonstrated that endothelial cells synthesize a plasma membrane protein indistinguishable from platelet glycoprotein (GP) IIa. The present study provides evidence for a further analogy between the platelet and the endothelial cell membrane by showing that cultured endothelial cells also synthesize a membrane protein complex immunologically related to the platelet GP IIb/GP IIIa complex. This evidence is based on the following observations: (1) C17, a murine monoclonal antiplatelet GP IIIa antibody, consistently precipitates two proteins, apparent molecular weights, respectively, 115,000 and 125,000 reduced and 95,000 and 135,000 nonreduced, from metabolically (^{35}S-methionine) as well as surface ^{125}I-labeled cultured human endothelial cells; (2) upon crossed immunoelectrophoresis of solubilized endothelial cells against a polyclonal rabbit antiplatelet antiserum and ^{125}I-labeled C17 IgG, a single precipitate of the protein(s) recognized by C17 is observed. As judged by their mobility in 9% polyacrylamide gels, both endothelial proteins appear to have a somewhat larger molecular weight than their platelet counterparts. Patterns obtained by crossed immunoelectrophoresis are also indicative of a difference in electrophoretic behavior of the platelet GP IIb/IIIa complex and the endothelial cell protein complex.

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ALTHOUGH IT IS BELIEVED that several processes related to thrombosis and hemostasis occur at the endothelial cell surface, little is known at present about the composition and architecture of the endothelial cell membrane. Attempts in our laboratory to produce monoclonal antibodies specifically directed toward endothelial cell membrane components, which could serve as tools to study the role of such membrane constituents in endothelial cell function, yielded several antibodies that reacted with platelets as well.[1]

Evidence was obtained using these monoclonal antibodies that endothelial cells synthesize a plasma membrane protein indistinguishable from platelet glycoprotein (GP) IIa.[1] In view of this observation, monoclonal antibodies against platelet GPs were tested for their ability to react with cultured endothelial cells. Here we show that one of these antibodies, designated CLB C17, directed to platelet GP IIIa,[2] recognizes an endothelial cell plasma membrane protein complex immunologically related to the platelet GP IIb/IIIa complex. Possible implications of this finding in terms of endothelial cell (patho)physiology are discussed.

MATERIALS AND METHODS

Preparation of endothelial cells and platelets. Culture, surface labeling with ^{125}I, and metabolic labeling with ^{35}S methionine of umbilical vein endothelial cells was performed as described.[1] Platelets from normal volunteers and from a previously described patient (R.E.) with Glanzmann's thrombasthenia[1] were prepared and surface-labeled with ^{125}I as outlined elsewhere.[1,2]

Immunoprecipitation. Proteins were precipitated from lysates of endothelial cells or platelets by preformed complexes of monoclonal antibodies and goat antimouse IgG as described in detail elsewhere.[1,2]

Crossed immunoelectrophoresis Crossed immunoelectrophoresis (CIE) of Triton X-100–solubilized platelets (Merck, Darmstadt, FRG) and endothelial cells against a rabbit antiplatelet antiserum, prepared as described,[3] was carried out as in Van Mourik et al and Kunicki et al,[1,3] except that an intermediate gel containing labeled monoclonal antibody was used in the second dimension.[4] The identity of the endothelial cell antigens present in the immunoprecipitate observed by CIE was established by direct sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis of ^{125}I antigens contained in individually excised precipitates as described,[3] except that indirect immunofluorescence was used to locate the antigen(s) recognized by the monoclonal antibody To this end, Triton X-100–solubilized ^{125}I-labeled endothelial cells were analyzed by CIE using an intermediate gel containing unlabeled monoclonal antibody. Gels were washed extensively but not stained. Bound monoclonal antibody was detected by overlaying the plates with FITC-labeled goat antimouse IgG

Monoclonal antibodies. Antibodies C17, C15, and 6C9 have been reported on elsewhere.[2,5] The epitopes recognized by these monoclonals reside probably on GP IIIa, as suggested by crossed immunoelectrophoresis and immunoprecipitation experiments.[2,5] Immunoblotting experiments, performed to better define the specificity of these antibodies, have thus far met with little success, however. We cannot fully exclude presently therefore, that these antibodies recognize epitopes that are specific for the GP IIb/IIIa complex, as suggested by Newman et al.[6] Antibodies H1 and Nx133 were also prepared in our laboratory and are all directed to epitopes on the GP IIb/IIIa complex based on immunofluorescence and immunoprecipitation studies with normal as well as thrombasthenic platelets. Details as to their specificity and effect on platelet function will be reported elsewhere.

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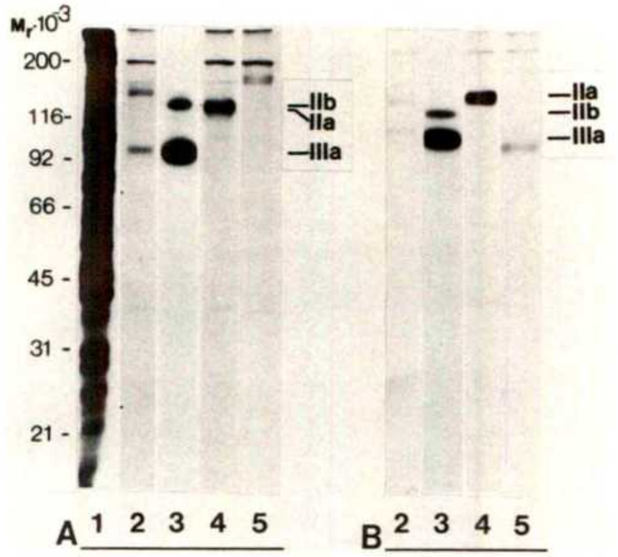

Fig 1. Immunoprecipitation of ^{35}S-methionine–labeled endothelial proteins with CLB-C17. Endothelial cells were cultured in the presence of ^{35}S-methionine for 24 hours as previously described.[1] Cell lysates were incubated with preformed complexes of monoclonal antibodies and goat anti-mouse immunoglobulins.[2] Washed ternary complexes were analyzed nonreduced (A) and reduced (B) by SDS-PAGE in 9% gels. Proteins were visualized by autoradiography. Lane 1, ^{35}S-methionine–labeled NP$_{40}$-solubilized endothelial cells. Lane 2, proteins precipitated with CLB-C17. For comparison, lane 3 shows ^{125}I-labeled IIb and IIIa precipitated with the same antibody from platelets; lane 4, ^{35}S-labeled IIa isolated with monoclonal antibody CLB-HEC 75[1]; and lane 5, an endothelial antigen precipitated by an endothelial cell-specific monoclonal antibody. (J.A. van Mourik et al, manuscript in preparation.)

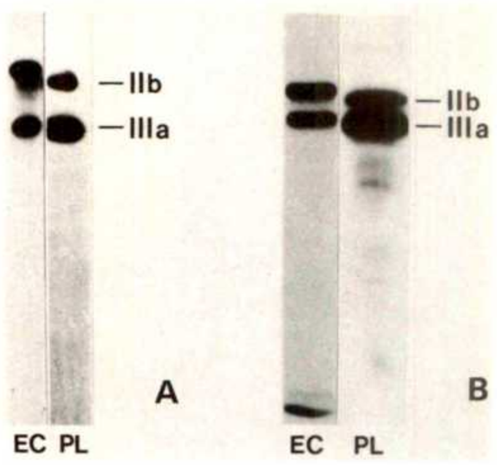

Fig 2. (A) Nonreduced and (B) reduced ^{125}I-labeled membrane proteins from endothelial cells (EC) and platelets (PL) recognized by CLB-C17. Endothelial cells and platelets were surface-labeled by the iodogen procedure, solubilized with NP$_{40}$, and incubated with complexes of antimouse IgG and CLB-C17. An autoradiogram of an SDS-PAGE (9%) analysis of the immunoprecipitates is shown.

RESULTS

Synthesis of a GP IIb/IIIa-like complex by cultured endothelial cells. Precipitation of GP IIIa from platelet lysates with monoclonal antibody C17 usually results in coprecipitation of GP IIb.[1,2] Incubation of lysates from either metabolically labeled (Fig 1) or surface-labeled (Fig 2) endothelial cells with preformed complexes of C17 and goat antimouse immunoglobulins also results in the precipitation of two labeled antigens that behave similarly to platelet GP IIb and IIIa upon SDS electrophoresis in 9% polyacrylamide gels under nonreducing and reducing conditions. Although it is apparent, especially in Fig 1, that other endothelial proteins have been trapped in the immunoprecipitate, the two proteins corresponding with platelets GP IIb and IIIa were specifically precipitated with antibody C17. The background pattern of trapped proteins was seen with other monoclonal antibody controls as well (Fig 1, lanes 4 and 5). However, the two bands representing endothelial IIb and IIIa were not observed in these control lanes. Furthermore, precipitation of the proteins of interest was prevented by the addition of unlabeled platelet lysate to the precipitation reaction (not shown). The metabolic labeling of GP IIb/IIIa was always less intense than that of GP IIa. Results from preliminary experiments (not shown) suggest that this is due to the fact that endothelial cells have more GP IIa than GP IIb/IIIa molecules on their surface.

CIE of solubilized endothelial cells. Upon CIE of solubilized platelets against a polyclonal rabbit antiplatelet antiserum and ^{125}I-labeled C17 IgG, the most predominant immunoprecipitation arc containing the GP IIb/IIIa complex (Fig 3A) can be visualized by autoradiography (Fig 3B). When an identical analysis is performed using an endothelial cell lysate, autoradiography also reveals a single radiolabeled precipitate (Fig 3C). This antigen has a lower mobility as compared with that of the platelet IIb/IIIa complex (Fig 3D). In contrast to what was observed for the platelet GP IIb/IIIa complex,[5] CIE patterns of the endothelial GP IIb/IIIa complex were unaffected by EDTA (not shown). The identity of the endothelial precipitate was established by SDS-PAGE analysis of the precipiated proteins (Fig 3E).

DISCUSSION

We have previously shown that endothelial cells and platelets share a membrane protein indistinguishable from platelet GP IIa.[1] Recently, Thiagarajan et al demonstrated that cultured endothelial cells also synthesize a platelet GP IIIa-like protein.[7] Using an immunoaffinity chromatography technique, which consistently resulted in the precipitation of GP IIb and IIIa from platelets, they were unable, however, to isolate GP IIb or another protein in stoichiometric amounts with this GP IIIa-like protein from endothelial cell lysates and therefore suggested that endothelial GP IIIa might not be complexed with a GP IIb-like protein. The present study does provide evidence that cultured endothelial cells synthesize a membrane protein complex, which appears to be similar to the platelet GP IIb/IIIa complex. Immunoprecipi-

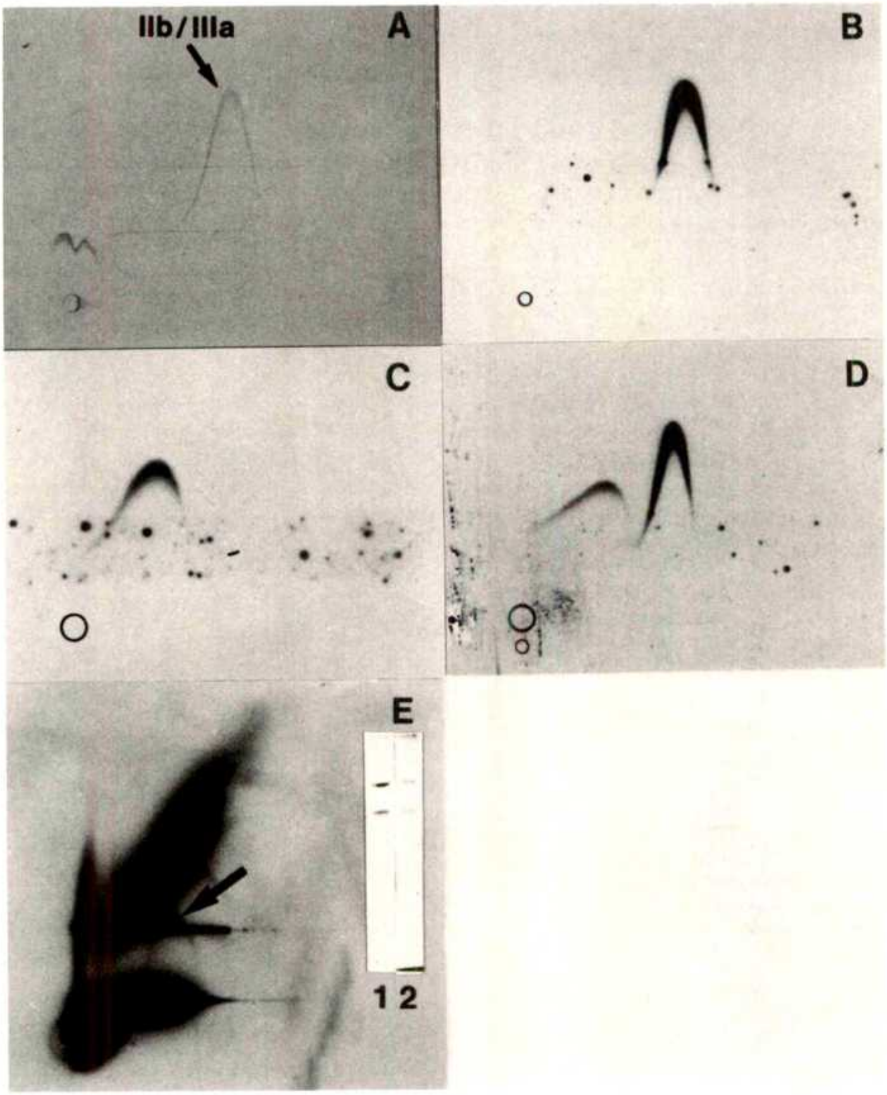

Fig 3. Crossed immunoelectrophoresis of Triton X-100–solubilized platelets and endothelial cells. Samples (equivalent to 1.5 × 10^7 and 2 × 10^6 cells, respectively) were electrophoresed in first-dimension gels containing 0.5% Triton X-100, and then in the second dimension against 0.85% (vol/vol) of a rabbit antiplatelet antiserum in the upper gel and ^{125}I-labeled CLB-C17 IgG in the intermediate gel. Slides were stained with Coomassie brilliant blue and subsequently subjected to autoradiography. (A) Coomassie stain of platelets. (B) Autoradiogram of (A). (C) Autoradiogram of endothelial cells. (D) Autoradiogram of platelets and endothelial cells (same amount of lysate of platelets and endothelial cells as applied in (B) and (C), respectively) run together on one plate. (E) Autoradiogram of ^{125}I-labeled endothelial cells. The location of the IIb/IIIa complex is indicated by a thick arrow. Inserts show an analysis by SDS-PAGE (9% gel, nonreduced) of the excized region encompassing the peak of the IIb/IIIa complex precipitate (lane 2). Lane 1 shows, for comparison, the proteins isolated from the same endothelial cell lysate by ordinary immunoprecipitation with antibody C17. Open circles indicate application wells (not visualized by autoradiography).

tation of GP IIIa with monoclonal C17 from endothelial cell lysates always resulted in the appearance of apparently stoichiometric amounts of GP IIb-like material in the precipitate. Furthermore, CIE experiments pointed toward the presence of a GP IIb/IIIa-like complex, which was recognized by a polyclonal rabbit antiplatelet antiserum. The precipitate obtained with this polyclonal antiserum could be radiolabeled by adding radiolabeled IgG of the nonprecipitating monoclonal C17 to the intermediate gel. These observations suggest that the platelet and the endothelial cell proteins share several epitopes.

Immunoprecipitation and CIE studies indicate that endothelial cells contain relatively little GP IIb/IIIa as compared with platelets, which is in keeping with the reported 100,000 GP IIIa-binding sites per endothelial cell.[7] In agreement also with the observation of Thiagarajan et al on endothelial GP IIIa,[7] endothelial and platelet GP IIb/IIIa appear to differ antigenically. We observed that only one, C17, of the five monoclonal antibodies against platelet IIIa or the IIb/IIIa complex (C17, C15, H1, 6C9, NX133) developed in our laboratory reacted with endothelial cells as judged by CIE. Immunoprecipitation studies suggest some reactivity of C15 with labeled endothelial cell proteins as two faint bands corresponding with the material precipitated by C17 were seen on autoradiograms of SDS-PAGE of C15 precipitates. The present study allows no conclusions as to the nature of these antigenic differences. Endothelial GP IIIa was suggested to have a slightly larger molecular weight than its platelet counterpart.[7] Our SDS 9% PAGE experiments point toward a similar difference for endothelial GP IIb. Furthermore, immunoprecipitation with C17 in the presence of EDTA at a high pH did not result, in contrast to what was observed with platelets,[2,5] in a preferential precipitation of GP IIIa. The C17 antigen from endothelial cell and platelet lysates also showed a difference in mobility as examined by crossed immunoelectrophoresis. To exclude that this difference was due to the presence of only endothelial GP IIIa within the endothelial precipitate, precipitation arcs obtained with ^{125}I-labeled endothelial cell lysates were excised and analyzed by SDS-PAGE.[3] Autoradiograms revealed the presence of both endothelial GP IIb and GP IIIa.

The observed differences between platelet and endothelial GP IIb/IIIa are not explained by the present study; it is an issue that clearly deserves further study as it may have considerable bearing on our conceptual idea regarding the role of endothelial GP IIb/IIIa in human (patho)physiology. For instance, in Glanzmann's thrombasthenia, platelets are deficient in glycoproteins IIb/IIIa,[8-10] the presence of an identical GP complex on endothelial cells could imply that this disorder is in fact an "endotheliopathy" as well. Since anti-IIb/IIIa antibodies have been observed in autoimmune thrombocytopenias,[11-13] the question whether such antibodies may also react with endothelial GP IIb/IIIa and perhaps contribute to the pathogenesis of petechiae in some of these patients is equally intriguing. Although binding of fibrinogen to endothelial cells has been observed,[14-16] additional functional studies will have to reveal whether endothelial GP IIb/IIIa indeed acts like platelet GP IIb/IIIa as a receptor for fibrinogen, von Willebrand factor, and fibronectin.[17-22] The answer to these questions, which are currently addressed in our institute, could lead to a better understanding of the nature of the endothelial GP IIb/IIIa complex.

NOTE ADDED IN PROOF

While this manuscript was under review, Fitzgerald et al[23] also reported the synthesis by human endothelial cells of membrane glycoproteins similar to platelet membrane GP IIb and IIIa.

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CONCISE REPORT

A New Translocation, t(10;14)(q24;q11), in T Cell Neoplasia

By Ian D. Dubé, Susana C. Raimondi, David Pi, and Dagmar K. Kalousek

Four cases of T cell neoplasia are reported: three presenting as T cell acute lymphoblastic leukemia and one presenting in the leukemic phase of a T cell lymphoma. In all cases, the cells of the leukemic clone were characterized by an identical cytogenetic abnormality. This abnormality was a unique reciprocal translocation involving chromosomes 10 and 14. The breakpoint in chromosome 14 was in band q11, coincident with the assigned locus of the α-chain gene of the T cell antigen receptor. The breakpoint in chromosome 10 was in band q24, a region reported to include the locus of the terminal deoxynucleotidyltransferase (TdT) gene. Our observations suggest that translocation t(10;14)(q24;q11) is specific for T cell neoplasia and that a gene in chromosomal band 10q24, possibly the TdT gene, plays an important role in T cell neoplasia when its expression or coding sequence is altered by aberrant recombination involving a T cell antigen receptor gene.

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EARLY CYTOGENETIC STUDIES in human cancer resulted in the identification of chromosomal changes that were specific for certain abnormal clonal proliferations (eg, Ph^1 in CML).[1] With the advent of improved cytogenetic techniques and better characterization of malignant cells, it became apparent that some cytogenetic changes were clearly of diagnostic[2] and more recently, prognostic[3] value. When molecular genetic techniques were used to investigate the basis of these cytogenetic changes, proto-oncogenes and "hotspots" for recombination were discovered at several of the nonrandomly involved chromosomal regions.[4]

In those cancers characterized by specific reciprocal translocations, a general pattern has emerged in which the expression or coding sequence of a proto-oncogene near one of the translocation breakpoints is altered as a result of its juxtaposition to a sequence of actively transcribed DNA from the other breakpoint. Knowledge of this pattern has been utilized in the cloning and characterization of putative oncogenes when only one sequence (that of the actively transcribed DNA) was known.[5,6]

Recently, T cell antigen receptor genes have been characterized.[7,8] It is now evident that T cell antigen receptor diversity is facilitated by the recombination of subunits in different parts of the genome.[9] The α-chain gene of the T cell antigen receptor has been assigned to chromosomal band 14q11 to q13.[10] This region is considered to be a hotspot for genetic recombination in differentiating T cells and has been observed to be nonrandomly involved in chromosomal abnormalities in T cell neoplasia.[11,12] A fragile site has been observed at chromosomal band 10q25,[13] and the gene for human terminal deoxynucleotidyltransferase (TdT) has recently been localized to the region 10q23-q25.[14]

We have observed four cases of T cell neoplasia in which the cells of the leukemic clones had in common a specific translocation involving chromosome 10 in band q24 and chromosome 14 in band q11. Our observations suggest that a gene, or genes, important in T cell proliferation and/or differentiation resides in 10q24 and can play an important role in T cell neoplasia. We speculate that the TdT gene may be involved in these translocations, becoming deregulated or mutated when juxtaposed to the α-chain gene of the T cell antigen receptor.

MATERIALS AND METHODS

Patients. Four patients with t(10;14)(q24;q11) were identified in a collaborative study that included 136 cases from Memphis, 28 from Toronto, and 52 from Vancouver. The translocation was observed at diagnosis in two of 164 cases of childhood acute lymphoblastic leukemia (ALL), at relapse in one of 52 cases of adult ALL, and at bone marrow relapse in one case of adult T cell lymphoma from a series of sporadically studied lymphomas. Of the 164 cases of childhood ALL, 22 were T cell ALL. (Routine immunophenotyping was not performed on the adult cases during the period of this study.) All four patients presented acutely without a history of prior hematological disorder. In cases 1, 2, and 3, the diagnosis of ALL was made on the basis of morphology and cytochemical staining of cells from peripheral blood and marrow. In case 4, the initial diagnosis of lymphoblastic lymphoma was made on the histopathology of a lymph node biopsy. Leukemic marrow cells from cases 1, 2, and 3 were tested for receptors to a host of immunologic markers. All patients were treated with intensive combined chemotherapeutic protocols. All patients were given CNS prophylaxis that included intrathecal methotrexate and received from five to seven chemotherapeutic agents intermittently for maintenance therapy. Informed consent for all biopsy specimens was obtained.

Cytogenetic studies. Marrow cells were obtained from heparinized posterior iliac crest aspirates, and the specimens were processed within 30 minutes of aspiration. Both direct and 24-hour cultures were done in all cases, except case 3 in which the marrow was processed by direct methods only. Cells in cases 1, 2, and 4 were studied using routine cytogenetic techniques.[15] Case 3 was studied by the method of Williams et al.[16] G-banded metaphase chromosomes were obtained according to routine techniques.[17] In all cases, a minimum of 25 metaphases were examined.

RESULTS

Patients. Partial clinical data on all patients are included in Table 1. Complete remission was achieved in all patients. Case 4 relapsed after eight months and died. Case 2 relapsed after four months, was successfully reinduced, but died shortly thereafter. Cases 1 and 3 have both been in

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Table 1. Partial Clinical and Hematologic Features in the Four Cases

Diagnosis	Case	Clinical Status	Age/Sex (yr)	Hgb (g/dL)	WBC (× 10⁹/L)	Platelets (× 10⁹/L)	% Blasts		Hepato/ Splenomegaly	Lymphadenopathy	CNS Involvement
							Blood	Marrow			
T cell ALL	1	Dx	5/M	5.4	180	15	80	80	+/+	+	−
	2	Dx	30/M	7.9	172	39	88	89	+/+	+	−
		Rel		10.7	129	48	3	92	−/−	−	−
	3	Dx	12/F	10.0	97	30	64	88	+/+	+	−
T cell lymphoma	4	Dx	53/M	13.6	9	311	1	18	−/−	+	−
		Rel		10.6	427	69	96	97	−/+	−	−

Abbreviations: Dx, diagnosis; Rel, relapse; M, male; F, female; Hgb, hemoglobin.

complete and continuous hematologic remission for eight months.

Cytochemical and morphological findings. Leukemic marrow cells in all four cases showed typical L1 morphology[18] and negative staining with Sudan black. Cases 1, 2, and 4 showed positive staining with periodic acid-Schiff and with acid phosphatase, while in case 3, staining was negative with periodic acid-Schiff and acid phosphatase was not done. TdT expression was studied only in case 3 where it was found to be expressed in 60% of marrow cells. This value was not significantly different from that observed in other childhood cases of T cell ALL (median, 62%, n = 21) or in childhood cases of non-T cell ALL (median, 63%, n = 115). Sections of the lymph node from case 4 revealed a diffuse infiltration of neoplastic lymphoid cells with a high nuclear to cytoplasmic ratio, convoluted nuclei, indistinct nucleoli, and a fine chromatin pattern. These findings were compatible with a T cell lymphoma.

Immunologic markers. The immunologic markers that characterized the respective leukemic clones were as follows: case 1, T4$^+$, T10$^+$, T11$^+$, Leu 1$^+$, AET$^+$, CALLA$^-$, Ia$^-$, Ba-2$^-$; case 2, Leu 1$^+$, Leu 2$^+$, Leu 6$^+$, AET$^+$, SIg$^-$; and case 3, T4$^+$, T8$^+$, T11$^+$, Ba-1$^+$, AET$^+$, CALLA$^-$, Ia$^-$, Pan T$^+$, Pan B$^-$, SIg$^-$. These findings confirmed that the leukemic clones in these patients represented T lineage clonal expansions.

Cytogenetic studies. Cytogenetic analysis of leukemic marrow revealed the following karyotypes; case 1, 46,XY (1 cell)/46,XY,t(10;14)(q24;q11),del(22)(q13) (24 cells); case 2, 46,XY (9 cells)/46,XY,−4,−17,+mar,+der(4)t(4;17)(q31;q21),t(10;14)(q24;q11) (8 cells)/45,XY,−4,−4,−17,+der(4)t(4;17)(q31;q21),+der(4)t(4;17)(q31;q21),t(10;14)(q24;q11) (8 cells); case 3, 46,XY,t(10;14)(q24;q11); and case 4, 46,XY,t(10;14)(q24;q11). The consistent cytogenetic abnormality was t(10;14)(q24;q11). Partial karyotypes from cases 1, 3, and 4 are shown in Fig 1.

DISCUSSION

We have presented four cases of T cell leukemia/lymphoma that have in common the following translocation in the leukemic clone; t(10;14)(q24;q11). While translocations involving the proximal long arm of 14 and the long arm of 10 have been reported in T cell neoplasia, breakpoints in chromosome 10 were either not defined or assigned to a band other than 10q24;[19,20] (Of particular interest is a cell line with a t(10;14)(q23;11.2) established from a patient with T cell lymphoma in relapse.[20]) Our report is thus the first report of this translocation occurring as a nonrandom cytogenetic abnormality in any human cancer. The translocation was found in both the pediatric and adult age groups. Except for the T cell immunophenotype and marked leukocytosis, there were no other clinical or laboratory features in this series that correlated with the presence of the t(10;14).

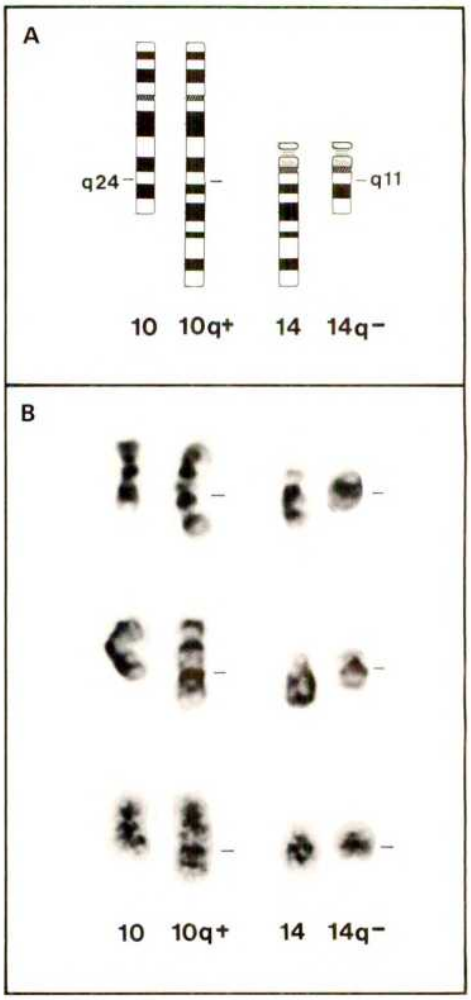

Fig 1. The 10;14 translocation associated with T cell neoplasia. (A) Diagram showing the G-banded pattern of rearranged chromosomes and homologues involved in the t(10;14)(q24;q11). (B) Representative partial karyotypes from three different patients with the t(10;14). Top, case 1; middle, case 4; and bottom, case 3. The lines indicate the approximate positions of the breakpoints.

The breakpoints observed in this translocation are intriguing. The one on chromosome 14 (q11) is in the region of the α-chain gene of the T cell antigen receptor[10] and is commonly[11,12] but not exclusively[21] involved in chromosomal rearrangements seen in T cell neoplasia. Based on the frequent observations in B cell neoplasia of translocations resulting in the juxtaposition, and subsequent deregulation, of the c-*myc* proto-oncogene with an immunoglobulin gene, it has been suggested that in T cell neoplasia, the T cell antigen receptor genes can assume the role played by the immunoglobulin genes in B cell neoplasia.[22] In support of this, Erikson et al have shown that the α-chain gene of the T cell receptor is split by a chromosomal translocation in two T cell leukemias.[23]

Several genes have been assigned to the distal long arm of chromosome 10,[24] including TdT.[14] The function of the TdT gene is not fully understood, but its expression is elevated in pre-B and pre-T cells.[25] It is also expressed at greatly elevated levels in lymphoblastic malignancies.[26] A common fragile site has been observed at 10q25,[13] and it has been suggested that fragile sites are predisposed to chromosomal mutation.[27] It is possible that the basis for the involvement of 10q24 in these four translocations lies simply in the generalized fragility of this region. It seems more likely, however, that a gene or genes important in T cell differentiation reside in 10q24. TdT may be such a gene and is a good candidate for playing a major role in T cell oncogenesis when its expression or coding sequence is altered as a result of juxtaposition to part or all of the α-chain gene of the T cell antigen receptor through aberrant recombination. Such an altered TdT that aberrantly or constitutively inserted nucleotides could be a potent carcinogen. Further studies are required to determine if TdT is different in cases of T cell neoplasia with the translocation compared to those without the t(10;14)(q24;q11).

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CONCISE REPORT

Use of Oligonucleotide Hybridization in the Characterization of a β^0-Thalassemia Gene (β^{37} TGG→TGA) in a Saudi Arabian Family

By Corinne D. Boehm, Carol E. Dowling, Pamela G. Waber, Patricia J. V. Giardina, and Haig H. Kazazian, Jr

Analysis of restriction site polymorphisms in the β-globin gene cluster of a Saudi Arabian female with β^0-thalassemia demonstrated that both of her β-globin genes were missing a nonpolymorphic *Ava*II site in exon 2. Examination of the normal nucleotide sequence surrounding this *Ava*II site revealed that either of two nucleotide substitutions, TGG→TAG or TGG→TGA, could produce a nonsense codon at codon 37 and eliminate the *Ava*II site. Consequently, two oligonucleotides (19-mers spanning codons 36 through 41 and containing either TAG or TGA at codon 37) were synthesized and hybridized against genomic DNA of the proband and her family. Specific hybridization with one of the oligomers demonstrated that the patient's β^0-thalassemia was the result of homozygosity for the TGG→TGA mutation at codon 37. In certain cases, oligonucleotide hybridization using genomic DNA may obviate the need for gene cloning and sequencing in the characterization of point mutations.

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THE USE OF synthetic oligonucleotide sequences for detection of single nucleotide differences in genomic DNA was pioneered by Wallace et al.[1,2] Oligomers have since been used for various diagnostic purposes, including prenatal detection of single-gene disorders such as α_1-antitrypsin deficiency,[3] sickle cell anemia and sickle hemoglobin C disease,[2,4] and certain β-thalassemias.[5-8] The discovery of a nonsense mutation in a factor VIII:C gene by use of oligonucleotide probes has recently been described.[9]

In the past decade, the molecular biology of the β^0- and β^+-thalassemias has been extensively studied. To date, 37 mutations have been described in peoples of Mediterranean, Middle Eastern, Asiatic Indian, and Pakistani, Chinese, Kurdish Jewish, and American black origin.[10-14] In general, each ethnic group has its own private set of β-thalassemia mutations, although exceptions, presumably due to more than one independent origin of the same mutation, have been observed.[10] To date, all point mutations producing β-thalassemia have been characterized by gene cloning and sequencing. Here we characterize a nonsense mutation in codon 37 of the β-globin gene by oligonucleotide hybridization.

MATERIALS AND METHODS

Subjects

The family studied is originally from Saudi Arabia. They deny any knowledge of consanguinity, although they are from the same village. They have hematologic values typical of β-thalassemia heterozygotes (mean corpuscular volume, mean corpuscular hemoglobin concentration, A_2 and hemoglobin values in the mother were 61.1fl, 31.3g/dl, and 7.8% and in the father were 65.4fl, 30.9g/dl, and 7%). The couple has two children with transfusion-dependent β^0-thalassemia and one child who is unaffected.

DNA Analysis

DNA was extracted by previously described methods from white blood cells of the mother, father, their daughter with β^0-thalassemia, and their homozygous normal daughter.[15] DNA was digested and Southern blots were prepared to examine β-globin region restriction site polymorphisms.[10,16] Haplotypes associated with the parental β^A- and β^{thal}-globin alleles were constructed through inheritance patterns observed in their affected and normal daughters.[17]

Oligonucleotide Analysis

Gel preparation and hybridization. After digestion, the DNA fragments were electrophoretically separated in a 1% agarose gel. Gels were prepared and hybridized with the end-labeled oligomers as previously described,[18] except that they were also prehybridized for 30 minutes at 37 °C in the hybridization buffer and then were hybridized with probe overnight at 55 °C (T_m minus 3 °C). Washes were as described,[18] except that a final two-minute wash in 2XSSPE at 56.5 °C (T_m minus 1.5 °C) was included. 1XSSPE is 10 mmol/L sodium phosphate pH 7.0, 0.18 mol/L NaCl, 1 mmol/L EDTA. The gel was first hybridized with oligomer B (Fig 1). After autoradiography, the gel-bound DNA was denatured, soaked in neutralization buffer, and rinsed in distilled water. The prehybridization and hybridization steps were repeated using oligomer A.

Probe preparation. Oligonucleotides were synthesized on an Applied Biosystems instrument. The full-length 19 nucleotide oligomer was further purified by electrophoresis through a 20% acrylamide gel of 1.5-mm thickness at 600 V for approximately six hours. After the 19-mer was excised from the gel, it was separated from acrylamide gel material by the crush-and-soak method.[18] Two hundred nanograms of each purified oligomer was incubated with 250 μCi of γ^{32}P-ATP (ICN No. 35020) and 3 to 5 units of T_4 polynucleotide kinase (New England Nuclear, No. NEE-101, Boston) at 37 °C for 40 minutes. The unlabeled oligomer was separated from the end-labeled product by polyacrylamide gel electrophoresis as described.[18] Typical specific activities were 3×10^8 cpm/μg of oligomer.

RESULTS

Polymorphism Haplotypes

To determine the feasibility of prenatal diagnosis for this couple, restriction site polymorphisms within and surrounding the β-globin gene were examined in the couple, one of their affected children, and a homozygous normal child. Haplotypes were determined using the following polymorphic restriction sites: *Hind*III within $^G\gamma$–, *Hind*III within $^A\gamma$–, *Hinc*II within $\psi\beta_1$–, *Hinc*II 3' to $\psi\beta_1$–, *Ava*II within $\psi\beta_1$–1, *Hinf*I 5' to β–, *Rsa*I 5' to β–, *Ava*II within β–, *Hind*III 3' to β–, and *Bam*HI 3' to β-globin genes. The

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Ava II recognition sequence:	GG ACC
Normal β-globin sequence, codon 36-1st nucleotide of codon 42:	CCT TGG ACC CAG AGG TTC T
Oligomer A:	CCT **TGA** ACC CAG AGG TTC T
Oligomer B:	CCT **TAG** ACC CAG AGG TTC T

Fig 1. Normal sequence of a portion of exon 2 of the β-globin gene. Also shown are the sequences of two 19-mer oligonucleotide probes, each of which contains a nonsense codon at the position homologous to codon 37 of the β-globin gene. Both nonsense codons destroy the *Ava*II recognition site that is normally present.

β^{A}-globin–associated haplotypes in both parents were $- - - - + + +$ (β^{A}) $+ + +$. Both parental β^{thal}-globin–associated haplotypes were $- - - - + + -$ (β^{thal}) $+ + +$.

Mutation in an Ava*II Site*

When genomic DNA from this family was digested with *Ava*II and probed with β-cDNA sequences, a new band appeared at 3.2 kb, unaccompanied by the loss of any bands normally seen (Fig 2a). This new band was present in the DNA of both parents and their affected daughter. It was absent from the DNA of their homozygous normal daughter. The appearance of the 3.2-kb fragment is consistent with the loss of a nonpolymorphic *Ava*II site in exon 2 of the β–globin gene (Fig 2b). The newly created fragment would have sequence in common with the β-globin cDNA probe being used (JW 102), which extends from codon 20 to the 3′ untranslated portion of the gene.[19] On the other hand, when the *Ava*II site in intact, the fragment lying between this site and the next *Ava*II site in the 5′ direction is not detected by the β-cDNA probe because it has only about 50 bp of sequence in common with this probe.

Oligonucleotide Analysis

Two oligonucleotide probes were synthesized, each of which could detect one of the two nonsense mutations that, if present, would destroy the *Ava*II site (Fig 1). The probes were 19-mers and included the sequences of codons 36 through 41 and the first nucleotide of codon 42. After digestion of genomic DNA with *Bam*HI, the β-globin gene fragment that includes codons 35 through 41 is 1.9 kb. After hybridization of *Bam*HI-digested genomic DNA with oligomer B, there was no signal at 1.9 kb with any of the genomic DNAs tested (Fig 3). This result indicated that TGG → TAG at codon 37 was not the mutation responsible for β°-thalassemia in this family. Subsequent hybridization of the same gel with oligomer A, which contained the nonsense codon TGA at the same position, revealed a 1.9-kb hybridizing fragment in the father, mother, and their affected daughter but not in their homozygous normal daughter nor in any of the control samples (Fig 3).

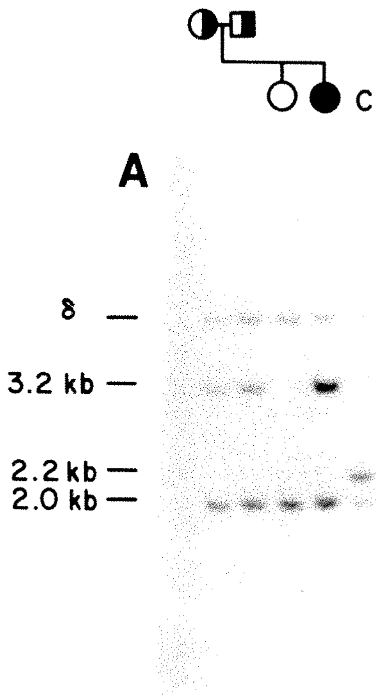

Fig 2. (A) Autoradiogram of *Ava*II-digested genomic DNA from family members indicated by the pedigree. β-Globin cDNA (JW 102) was used as the probe. The two alleles of an *Ava*II polymorphism in IVS 2 of the gene appear as 2.2-kb and 2.0-kb fragments on the autoradiogram. All family members are homozygous for the presence of the polymorphic site (+/+), and the control (c) is heterozygous +/−. A new band at 3.2 kb is seen in this family and is segregating with the β-thalassemia allele. The appearance of this fragment indicates loss of a nonpolymorphic *Ava*II site in exon 2 of the β-globin gene. Probe cross hybridizes with δ-globin sequences. (B) *Ava*-II restriction map of a normal β-globin gene and the mutant β-globin gene described in the text. Solid arrows (↑) indicate the locations of the nonpolymorphic *Ava*II restriction sites within and flanking the β-globin gene. Solid arrows with asterik (↑*) indicate the location of a polymorphic *Ava*II site, which is present in both the mutant and normal β-globin genes. Open arrow (↑) indicates the location of the nonpolymorphic *Ava*II site, which is destroyed in the mutant gene. The 3.0-kb fragment is not seen in the autoradiogram in Fig 2a because the 5′ end of the JW 102 β-globin cDNA probe is complementary to only about 50 bp of this fragment.

DISCUSSION

Coding region mutations producing β-thalassemia are of several types, including frameshift mutations due to small deletions or insertions, mutations that affect cryptic splice sites, or chain terminator (nonsense) mutations.[10] Any of these can cause loss of an endonuclease restriction site.[20-23] In addition, clinically insignificant, or neutral, nucleotide substitutions within coding regions can also eliminate an endonuclease restriction site.[24,25] In the case described here, DNA restriction analysis indicated that a nonpolymorphic *Ava*II site in exon 2 of the β-globin gene was absent in a β°-thalassemia allele. Both codons 37 and 38 are part of the *Ava*II recognition sequence (GGACC) that is lost in this β°-thalassemia allele (Fig 1). An insignificant substitution in the third nucleotide of codon 38 (ACC → ACT, ACA, or ACG) could be responsible for the loss of the *Ava*II site and be unrelated to the mutation producing β°-thalassemia. In contrast, a frameshift mutation due to insertion or deletion of nucleotides in codon 37 or 38 or both could eliminate the

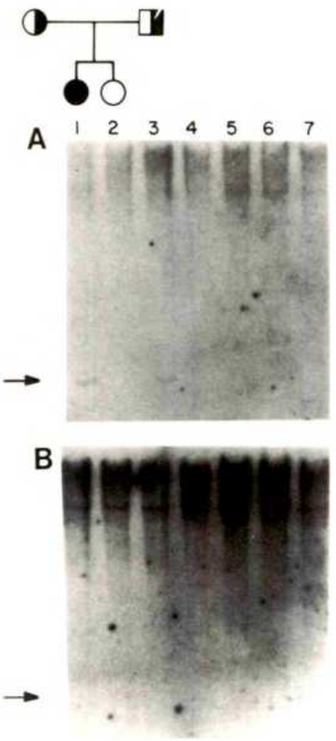

Fig 3. **Autoradiograms of *Bam*HI-digested genomic DNA from family members shown in the pedigree. The gel was hybridized initially with oligomer B (panel B), which contains the TAG nonsense codon, and then subsequently hybridized with oligomer A (panel A), which contains the TGA nonsense codon (see Materials and Methods). Arrows in panels A and B indicate the location of the 1.9-kb *Bam*HI fragment in which codon 37 resides. Lanes are (1) β^0-thalassemia patient described here, (2) her β^A/β^A sister, (3) her β^A/β^{thal} father, and individuals (4) homozygous for nonsense codon 39 mutation, (5) heterozygous for nonsense codon 39 mutation, and (6) and (7) homozygous for normal β-globin alleles. Hybridization with DNA of the β^A/β^{thal} mother was readily visible with oligomer A but was too weak to be seen in the reproduction, and that lane has been omitted from the figure.**

*Ava*II site and produce β^0-thalassemia. Such a mutation would be difficult to characterize without cloning and sequencing the affected gene. However, the *Ava*II site could also be lost by either of two single nucleotide changes, producing nonsense mutations in codon 37 (TGG to either TAG or TGA). Oligonucleotide hybridization was chosen as a method with which we could determine relatively easily whether or not one of these nonsense mutations was present. Oligomer A identified the presence of a single nucleotide change G → A in codon 37, producing a TGA nonsense mutation.

This nonsense 37 mutation has not been observed among 400 or so Mediterranean, Indian, and Oriental β-thalassemia genes that we have examined by *Ava*II analysis with the JW102 β-globin probe. The molecular basis of β-thalassemia in Saudi Arabians, however, has not been extensively studied. Thus the frequency of the nonsense 37 allele in this population is not known. Although consanguinity is not known to be the case in this family, it may be the reason for the homozygous presentation of this allele.

The polymorphism haplotype associated with the nonsense 37 mutation is a subset of the haplotype I described in Orkin et al[24] and is a haplotype commonly associated with β^A-globin alleles. The parental β^A-associated haplotypes are identical with one another and differ from the nonsense 37-associated haplotype only at the *Rsa*I site 5′ to the β-globin gene.

NOTE ADDED IN PROOF

The gel used in Fig 3 was subsequently hybridized with an oligonucleotide probe corresponding to normal sequence at codons 36 through 41. DNA from the affected girl did not hybridize to this oligonucleotide probe, while that of other family members demonstrated a band at 1.9 kb. This indicates that the patient is homozygous for the β^{37} mutation.

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CONCISE REPORT

The Cofactor Role of Protein S in the Acceleration of Whole Blood Clot Lysis by Activated Protein C In Vitro

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The effect of purified human activated protein C (APC) and protein S on fibrinolysis was studied by using an in vitro blood clot lysis technique. Blood clots were formed from citrated blood (supplemented with ^{125}I-fibrinogen) by adding thrombin and Ca^{2+}-ions; lysis of the clots was achieved by adding tissue-type plasminogen activator. The release of labeled fibrin degradation products from the clots into the supernatant was followed in time. We clearly demonstrated that APC accelerates whole blood clot lysis in vitro. The effect of APC was completely quenched by anti-protein C IgG, pretreatment of APC with diisopropylfluorophosphate, and preincubation of the blood with anti-protein S IgG. This demonstrates that both the active site of APC and the presence of the cofactor, protein S, are essential for the expression of the profibrinolytic properties. At present, the substrate of APC involved in the regulation of fibrinolysis is not yet known. Analysis of the radiolabeled fibrin degradation products demonstrated that APC had no effect on the fibrin cross-linking capacity of factor XIII.

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PROTEIN C is a vitamin K-dependent plasma protein that, on activation by thrombin/thrombomodulin, exerts anticoagulant activity by inactivating coagulation factors Va and VIIIa.[1,2] Protein S, another vitamin K-dependent plasma protein,[3] serves as a cofactor for activated protein C (APC) in expressing this anticoagulant effect.[4,5]

Several investigators presented evidence that APC is not only a potent anticoagulant, but also has profibrinolytic properties (both in vitro and in vivo).[6-9] Comp and Esmon[6] reported increased plasma plasminogen activator activity after infusion of bovine APC into dogs, which they considered to be a result of stimulation of tissue-type plasminogen activator (t-PA) release from the vessel wall by a secondary messenger triggered by APC. However, Colucci et al[7] could not establish any increase of fibrinolytic activity in squirrel monkeys, neither after administration of human APC nor thrombin–thrombomodulin complex. In vitro, Taylor et al[8] reported that APC enhances blood clot lysis.

So far no experimental data have been presented on whether protein S serves as a cofactor for APC in its profibrinolytic effects. In the present study, we investigated a possible role of human APC and protein S in t-PA–dependent fibrinolysis in vitro by means of a whole blood clot lysis method. Complete lysis of the whole blood clot was achieved within three hours by the addition of purified human t-PA. The results clearly demonstrate that APC enhances blood clot lysis in vitro and that protein S is an essential cofactor for this effect of APC. A preliminary report of these studies has been presented the Tenth International Congress on Thrombosis and Haemostasis, San Diego.[9]

MATERIALS AND METHODS

Whole blood clot lysis. Blood from healthy donors was collected by venipuncture in plastic tubes in 1/10 vol of 0.11 mol/L sodium citrate and supplemented with 7.5 μL of ^{125}I-labeled fibrinogen solution per milliliter, 30 or 60 IU t-PA/mL, APC (final concentration 7.75, 15.5, 46.5 nmol/L), and a vol of 0.15 mol/L NaCl to obtain a final blood concentration of 80%. After rapid mixing, 250 μL aliquots were transferred to Eppendorf cups containing 3 μL of 60 μmol/L thrombin solution and 2.5 μL of 1.25 mol/L CaCl$_2$. Immediately after mixing, the cups were incubated at 37 °C to allow clot formation and subsequent lysis. Clot formation occurred within 60 seconds. At that time, 90% of the ^{125}I-fibrinogen was incorporated into the clot. At different time intervals, tubes were centrifuged in a MSE Micro Centaur centrifuge (Beun de Ronde, Amsterdam) for five minutes 11.50 g (4 °C). The supernatant (100 μL) was mixed with 100 μL of a solution containing 2% sodium dodecyl sulfate (SDS), 20% glycerol, 6 mol/L urea, and 0.1 mol/L Tris/HCl buffer, pH 6.8, counted for radioactivity (^{125}FDP[fibrin degradation products] in a gamma counter (Packard 5110P, BC Netherlands) and analyzed by SDS-polyacrylamide gel electrophoresis, according to Laemmli.[10] Clot lysis was expressed as x-b/t-b × 100%, in which x = radioactivity of the supernatant, b = radioactivity of the blanc, t = total radioactivity added before clotting. The blanc value (<10%) is the radioactivity of the supernatant of whole blood 30 minutes after clotting and without addition of t-PA. One hundred percent lysis indicates complete clot lysis. After electrophoresis, autoradiography of the slab gels was carried out with a Kodak-X-ray Omat AR film (Kodak X-omatic regular) for 72 hours. Quantitatively similar results were obtained with blood of different donors at both t-PA concentrations.

Fibrinogen (Fbg) was isolated from human plasma, as described previously,[11] and labeled with ^{125}I by the iodogen method.[12] The molar ratio Fbg/iodine was approximately 2. The amount of radioactivity not clottable with thrombin was less than 10% of the total radioactivity. The radiolabeled Fbg, dissolved in 0.15 mol/L NaCl, had a concentration of 2 mg/mL and a specific radioactivity of 75.10^6 cpm/mg.

Human alpha (α) thrombin was prepared and purified as described previously.[13]

The two-chain form of t-PA was isolated from human (Bowes) melanoma cells and purified as described.[14] This activator is considered to be identical with the plasminogen activator in blood after its release from the vessel wall.[15]

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Protein C was purified and activated as described previously.[13,16] Fibrinolytic activity could not be detected in the APC preparation, neither on plasminogen-rich fibrin plates[17] nor in a t-PA assay with the chromogenic substrate S-2251 (Kabi Vitrum, Stockholm).[18] APC was inactivated with diisopropylfluorophosphate (DFP) or with anti-protein C IgG as described previously.[19] One milliliter of anti-protein C IgG (2 mg/mL) completely neutralizes the protein C activity of 25 mL of pooled normal plasma within 15 minutes.

Neutralization of blood protein S with antiprotein S IgG. Blood was incubated for 15 minutes at 37 °C with antiprotein S IgG (100 μg/mL) prepared from rabbit antiserum against purified human protein S.[20] Under these experimental conditions, 1 mL of anti-protein S IgG (2.0 mg/mL) neutralizes the APC-cofactor activity of the protein S of 10 mL of pooled normal plasma completely. Antiprotein S IgG did not influence the APC activity as was measured by an amidolytic assay using S-2366.

RESULTS

Figure 1 shows that APC, added to the blood before clot formation was induced by thrombin and Ca^{2+}, greatly accelerates the lysis rate of the clot and that the effect increases with higher APC concentrations. Similar results were obtained with blood that had not been anticoagulated with citrate (data not shown). Because the lysis of blood clots from different donors showed different lysis rates at the same concentrations t-PA, the effect of APC was studied routinely at two t-PA concentrations (30 and 60 IU/mL). At both concentrations, APC had an accelerating effect on clot lysis. APC pretreated with anti-protein C-IgG or inactivated by DFP (Fig 2) no longer accelerated the lysis of blood clots. To neutralize all endogenous protein C, blood was preincubated with anti-protein C IgG, clotted, and lysed as described. A small inhibitory effect on the lysis rate was observed compared with normal blood (Fig 3). This indicates that part of the endogenous protein C, when activated by thrombin to APC, might contribute to the rate of clot lysis in vitro.

To investigate whether protein S plays a role in the acceleration of clot lysis by APC, blood protein S was neutralized before clotting with antiprotein S IgG. The effect of APC on clot lysis was completely quenched when all protein S was inactivated (Fig 4). Incubation of blood with nonimmune rabbit IgG had no effect on clot lysis either in the absence or presence of APC (not shown). To study whether APC has any effect on the cross-linking capacity of factor XIII, and therefore accelerates clot lysis,[21] the radioactive FDP formed in the supernatants were separated on SDS-polyacrylamide gels and visualized by autoradiography. A D-dimer band and degradation products with a higher molecular weight than fibrin (340 kd) were identified as cross-linked FDP. No differences in electrophoresis patterns of FDP from clots lysed with and without APC present were found when compared on the basis of an equal percentage of lysis. This indicates that the extent of cross-linking by factor XIII was not influenced by APC (data not shown).

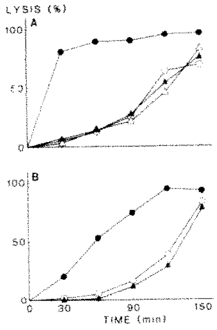

Fig 2. Effect of APC inactivated by pretreatment with anti-protein C IgG (A) and with DFP (B) on the lysis rate of blood clots. Citrated whole blood from healthy donors (A: A.v.W.; B: F.H.) was clotted and lysed as described in Materials and Methods. Thirty International Units t-PA per milliliter of blood was used. Curves represent lysis without addition of APC (○), after addition of 46.5 nmol/L APC (●), 46.5 nmol/L APC pretreated with antiprotein C IgG (△), 75 μg anti-protein C IgG/mL blood (▲, A), and of 16 nmol/L APC pretreated with DFP (▲, B).

DISCUSSION

In the present study, we demonstrated by means of a t-PA-dependent whole blood clot lysis method that human APC added before clotting greatly accelerates the lysis rate of whole human blood clots in vitro. Active APC is required for this effect, since APC inactivated with antiprotein C IgG or DFP did not show an accelerating effect on clot lysis. Several other investigators have suggested a possible role for APC in fibrinolysis.[6-9,19,22,23] An acceleration of blood clot lysis in vitro has been previously reported by Taylor et al.[8] In their experiments, complete lysis was achieved during incubation periods up to 30 hours. This may not be an optimal

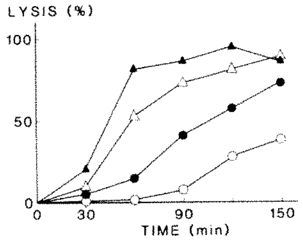

Fig 1. Influence of APC on the lysis of human blood clots in vitro. Citrated whole blood from a healthy donor (P.K.) was clotted and lysed as described in Materials and Methods. Thirty International Units t-PA per milliliter of blood was used. Curves represent lysis without addition (○) and with 7.75 nmol/L (●), 15.5 nmol/ (△), 46.5 nmol/L (▲) APC.

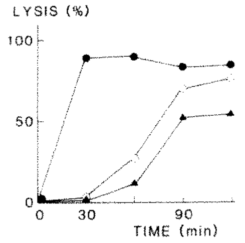

Fig 3. Contribution of endogenous APC to the acceleration of blood clot lysis. Citrated whole blood from a healthy donor (A.v.W.) was incubated for 15 minutes at 37 °C with antiprotein C IgG (75 μg/mL) (▲) or saline (○, ●) clotted and lysed as described in Materials and Methods. Sixty International Units per milliliter t-PA was used. Curves represent lysis without addition of exogenous APC (○, ▲) or after addition of 46.5 nmol/L APC (●).

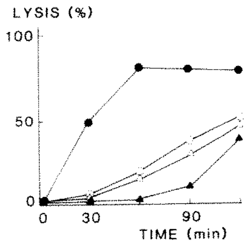

Fig 4. Effect of the elimination of protein S on the APC-induced acceleration of whole blood clot lysis. Citrated whole blood from a healthy donor (F.H.) was incubated for 15 minutes at 37 °C with antiprotein S IgG (100 μg/mL) (triangles) or saline (circles) clotted and lysed as described in Materials and Methods. Sixty International Units t-PA was used. Curves represent lysis without addition of APC (○, ▲) and after the addition of 46.5 nmol/L APC (●, △).

condition for correlation changes in lysis rate with APC concentrations, since the half-life of APC in blood is about 20 minutes, due to the presence of a protein C inhibitor.[24]

So far no data have been published on the role of protein S in fibrinolysis. From previous studies, it is known that the prolongation of the activated partial thromboplastin time by APC is dependent on the presence of protein S, and that the inactivation of factor Va by APC depends on the presence of phospholipids and Ca^{2+} ions and is greatly accelerated by the addition of protein S.[25] Walker[5] proposed that protein S increases the affinity of APC for binding to phospholipid membranes, and thus accelerates the proteolytic degradation of factor Va on the phospholipid surface. In this report (Fig 4), we demonstrate that protein S is an obligatory cofactor of APC for its accelerating effect on in vitro fibrinolysis. This observation also suggests that procoagulant phospholipids, which become available by platelet activation, might be involved in the action of APC on the fibrinolytic system.

At present, no definitive information is available on the mechanism by which APC accelerates t-PA–dependent fibrinolysis. Because no differences were found in electrophoresis patterns of FDP from clots lysed with and without APC present, it seems unlikely that APC interferes with the cross-linking capacity of factor XIII. Another possible mechanism might be that APC inactivates an inhibitor of t-PA, and thus increases the concentration of active t-PA. Indeed, we and others found that APC decreases plasminogen activator activity in endothelial cell-conditioned medium.[19,22] However, the complexity of the clot lysis system does not permit us to extrapolate our data to those obtained in the t-PA inhibitor experiments in endothelial cell culture systems. Taylor and Lockhart[23] suggested that APC inhibited a plasminogen activator inhibitor released by phorbol diester-stimulated mononuclear cells, thus inducing acceleration of fibrinolysis.

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CORRESPONDENCE

LEFT SHIFT OF PERIPHERAL BLOOD COUNT AT DIAGNOSIS OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

To the Editor:

The presence of myeloid precursors in the blood (left shift) is a well-recognized presenting feature in some cases of childhood acute lymphoblastic leukemia (ALL).[1] Shen et al,[2] in a retrospective analysis of 109 children with ALL, found that left shift (in 43% of the patients) was correlated with longer durations of complete remission. The authors postulated that this feature may be an indirect measure of total leukemia cell burden, reflecting less suppression of granulopoiesis. Otherwise, very little is known about the clinical importance of left shift in the peripheral blood.

Between May 1979 to December 1983, 427 children with newly diagnosed ALL were admitted to St Jude Children's Research Hospital. They were subjects of a Total Therapy study that incorporated three different treatment protocols based on the patients' initial risk features and subsequent randomization. The details of risk assignment and treatment have been previously published.[3] Seventy-four patients (17.3%) were found to have a left shift in their blood counts, defined by the presence of 1% or more of metamyelocytes, myelocytes, or promyelocytes in a differential count of 100 cells. Children with left shift were also more likely to have circulating nucleated red blood cells (20 of 74 patients versus 35 of 353, $P < 0.001$). By comparison with common ALL, cases of T cell and so-called "undifferentiated" ALL had higher frequencies of left shift: 44 of 304 (14%), 15 of 59 (25%), and 8 of 26 (31%), respectively ($P = 0.02$). The presence or absence of left shift was not related to age, sex, race, French-American-British (FAB) morphologic subtype, percentage of S-phase cells, blast cell DNA index, liver or spleen size, initial leukocyte count, percentage of blasts in bone marrow, platelet count, hemoglobin level, or serum lactic dehydrogenase level. More importantly, with a median follow-up time of 4 years 2 months, left shift had no impact on treatment outcome whether the analysis was performed on combined (Fig 1) or separate protocol groups.

The reason for the lower percentage of patients with left shift in our series compared with that of Shen et al[2] is not clear. Nonetheless, we were unable to demonstrate any association between left shift and leukemia cell burden as reflected by leukocyte count, liver or spleen size, percentage of blasts in bone marrow, and serum lactic dehydrogenase level.[4] The similarity of relapse-free survival times for patients with or without left shift in our study contrasts with the results of Shen et al.[2] These findings indicate that left shift is a relatively unimportant presenting feature and support the idea that prognostic factors in childhood ALL can differ with changes in therapy.[5,6]

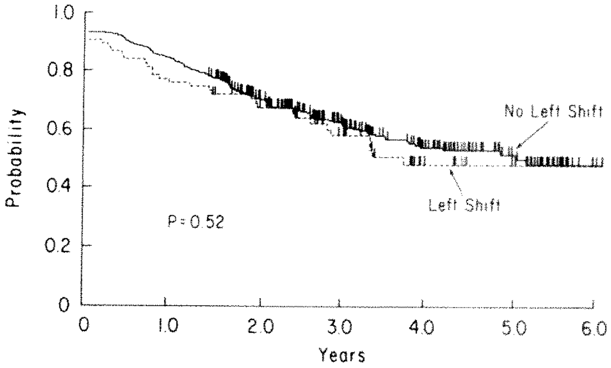

Fig 1. Life-table probability of disease-free survival according to the presence or absence of a left shift in peripheral blood counts among children treated for ALL.

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To the Editor:

There are two major differences between the findings of Pui et al and our own. The first and obvious one is that left shift (LS) in their series is not a prognostic indicator. The second is the presence of LS in 47/109 of our patients, but only 74/427 of their patients. Clearly, then, the patient subpopulation defined as having LS is markedly different in the two studies. There are a number of ways in which this may have arisen. Morphologic interpretation was subjective, and was done by light microscopy in our series. We do not know how it was done in the St Jude study. Perhaps the differential counts were performed on automated equipment. Our patient referral pattern is different from the St Jude one. We are an urban center, not a national referring institution. Perhaps our patients are admitted

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earlier than in St Jude's, and their leukemic cell load may be less. In our recent leukemia study, 182/230 patients presented with a white cell count at diagnosis of $< 30 \times 10^9/L$ and 115 had a white cell count of $< 10 \times 10^9/L$. There are, of course, other possibilities such as sample size differences, or the different intensity of chemotherapy treatment eliminating the prognostic value of LS.

Our findings on the prognostic value of LS led us to undertake in vitro studies on the effect of bone marrow leukemia cell underlayers on the growth of normal bone marrow in the presence and absence of leukocyte conditioned media (LCM) in microcell agar culture. We found growth of normal marrow and leukemia underlayers without LCM in 7/12 underlayers prepared from patients with LS, but in only two out of eight prepared from underlayers of patients with ALL and no LS ($P = 0.09$). There is therefore some in vitro experimental evidence that the interaction between leukemia blasts and normal stem cells may be very complicated and variable, and that LS in the peripheral blood may be an indirect indicator of this balance.

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ANNOUNCEMENTS

PETER DEBYE-PRIZE 1987 IN CARDIOVASCULAR DISEASES

The University of Limburg at Maastricht, The Netherlands, has been given the opportunity to award the Peter Debye-prize. This prize, in the amount of 20,000 guilders, is an award of appreciation. The funds for the Peter Debye-prize are provided by the Edmond Hustinx Foundation, founded by the Maastricht industrialist Edmond Hustinx, who was greatly interested in promoting science and culture. The prize is named after the physicist Peter J.W. Debye (1884–1966), a native of Maastricht, who was awarded the Nobel Prize for chemistry in 1936.

In January 1987 the prize will be presented for the fifth time, to a person or group of persons (three persons maximum) considered to have made a fundamental contribution to research in the field of cardiovascular diseases, particularly in molecular-chemical aspects, including arteriosclerotic processes, blood coagulation, ischemia, reperfusion, and chemical kinetics of substrates, ions, and macromolecules in the heart.

An international jury has been formed for the awarding of this prize. This jury would like its attention drawn to persons or groups of persons from any country to be considered for the award on the basis of their scientific work in the area indicated.

Nominations (in English) should include a curriculum vitae, a survey of the scientific achievements of the candidate(s) (not exceeding four pages), and a list of publications. The deadline for nominations is September 1, 1986. Further information about the prize is available from Dr. E.H.S. Drenthe, Secretary of the Jury, Office of the Rector, University of Limberg, PO Box 616, 6200 MD Maastricht, The Netherlands.

EXECUTIVE DIRECTOR

An Executive Director is being sought for the Puget Sound Blood Center in Seattle, Washington. The PSBC is a centralized regional blood banking facility that employs 240 people, including seven physicians, and is responsible for the distribution of 250,000 units of blood components annually. Candidates must have an MD degree and have demonstrated administrative skills in transfusion medicine and the ability to contribute to and broaden the ongoing research activities of the Center. The position carries a senior faculty appointment in the School of Medicine at the University of Washington. Interested candidates should send their CV and bibliography as well as the names of three references to Dr. Daniel H. Coleman, c/o Search Committee, Puget Sound Blood Center, Terry and Madison Streets, Seattle, Washington 98104. The PSBC is an Equal Opportunity Employer.

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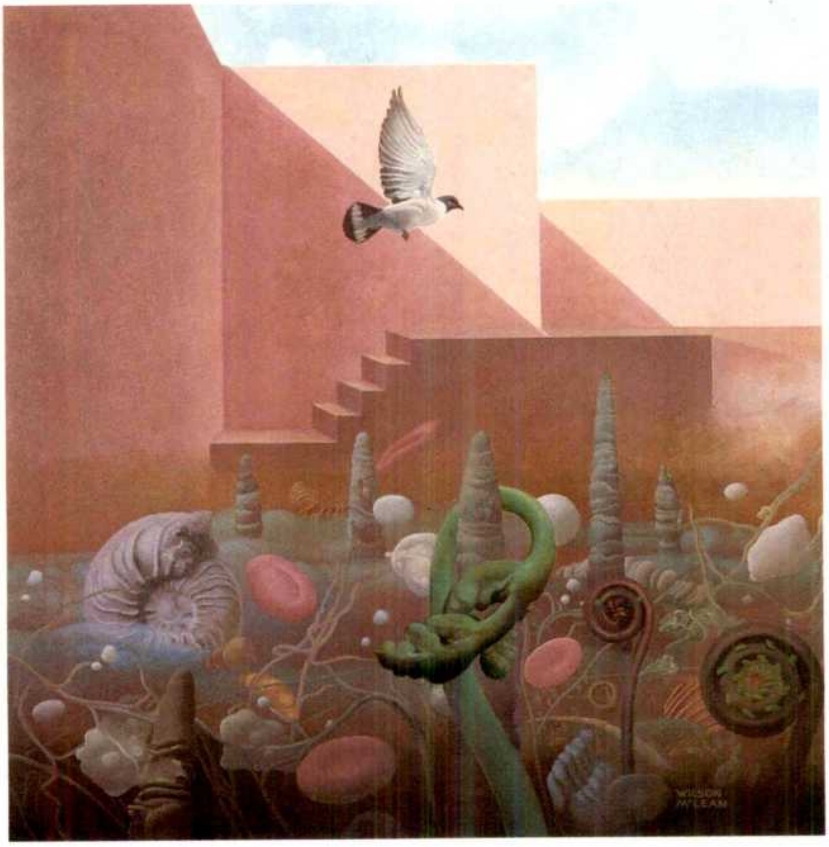

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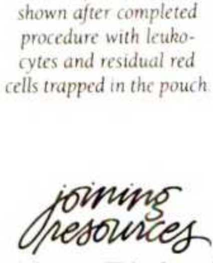

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HERPES SIMPLEX INFECTION IN THE COMPROMISED HOST:

OFTEN SERIOUS, TOO OFTEN UNSUSPECTED

Number one in a series of typical case presentations.

Esophagitis in a leukemia patient

In patients with hematologic malignancies, the visceral organ most frequently involved by herpes simplex virus (HSV) infection is the esophagus.[1] Extensive ulcerative lesions may develop on the upper two thirds of the esophagus with confluent lesions of the distal third.[2] In some cases, however, the mucosa may be grossly unremarkable even when microscopic examination shows evidence of herpetic ulcerative esophagitis.[1] Similarly, some patients may display symptoms of dysphagia and burning retrosternal pain,[3] whereas others may appear asymptomatic.[1] The diagnostic method of choice for patients with suspected herpetic esophagitis is esophagoscopy with visualization of the mucosa and procurement of cytologic and biopsy specimens from ulcerated areas.[1]

References: 1. Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **2.** Wong KK, Hirsch MS: Herpes virus infections in patients with neoplastic disease. Diagnosis and therapy. *Am J Med* 1984; 76:464-478. **3.** Lightdale CJ, Wolf DJ, Marcucci RA, et al: Herpetic esophagitis in patients with cancer: Ante mortem diagnosis by brush cytology. *Cancer* 1977;39:223-226.

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Herpetic infections may result in extensive disease and death

Immunocompromised patients are highly vulnerable to HSV infection. Such infections may interrupt therapy and even lead to death if not detected early and managed aggressively.[1] Although the initial appearance may be mild, HSV infection may progress to severe localized mucocutaneous ulcerations. The greatest threat that herpes simplex poses is an infection that disseminates from a mucocutaneous or unrecognized primary lesion and involves the lungs, liver, esophagus, or central nervous system.[1] Furthermore, herpetic ulcers frequently provide an entry for secondary bacterial and fungal pathogens.[2]

Infections may not be diagnosed correctly without virologic study

Some investigators claim that the actual incidence of serious HSV infection is probably higher than reported because "the possibility of herpetic disease is insufficiently considered and appropriate diagnostic viral studies are often not carried out."[3] One study, for example, found histocytologic evidence of visceral herpes infection in 56 patients even though herpetic involvement was not suspected in any of the patients prior to autopsy.[2]

ZOVIRAX I.V. halts viral replication and speeds healing even in debilitated patients

ZOVIRAX I.V. has been shown to be a highly effective treatment for severe mucocutaneous HSV infection in immunocompromised patients. In controlled trials, ZOVIRAX I.V. rapidly halted viral shedding — within 3 days in most patients — and significantly shortened healing time as well as duration of pain.[4,5] Furthermore, ZOVIRAX I.V. may allow patients to return home sooner and enable patients with intraoral lesions to resume normal eating habits.[6]

ZOVIRAX I.V. is well tolerated by immunocompromised patients

A multicenter collaborative trial of ZOVIRAX I.V. in immunocompromised patients reported that: "Clinical benefits were obtained without appreciable toxicity. The most common reactions were irritation of peripheral veins used for drug infusion and a low incidence of rash.... Significant renal or hematologic toxicity did not occur, even in these seriously ill patients who were commonly receiving other drugs concomitantly."[5]

Note: Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes. Please see brief summary for more information.

References: 1. Feldman S, Cox F: Viral infections and hematological malignancies. *Clin Haematol* 1976;5:311-328. **2.** Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **3.** Muller SA, Herrmann EC, Winkelmann RK: Herpes simplex infections in hematologic malignancies. *Am J Med* 1972;52:102-114. **4.** Wade JC, Newton B, McLaren C, et al: Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation. *Ann Intern Med* 1982;96:265-269. **5.** Meyers JD, Wade JC, Mitchell CD, et al: Multicenter collaborative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. *Am J Med* 1982;73(1A):229-235. **6.** Data on file, Burroughs Wellcome Co.

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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously, or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: 1. C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** J.C. Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am. J. Med. 73(1A): 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. 33:1452-1463, 1973.

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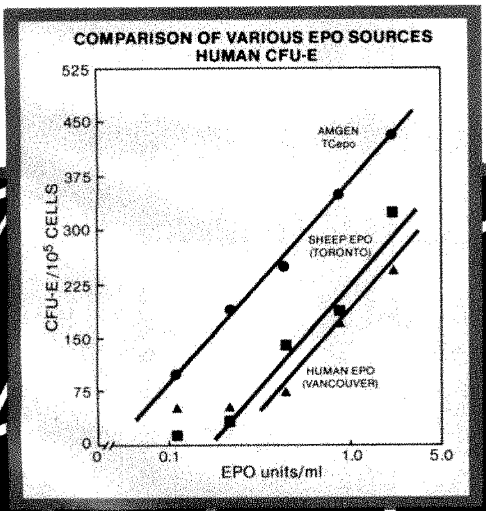

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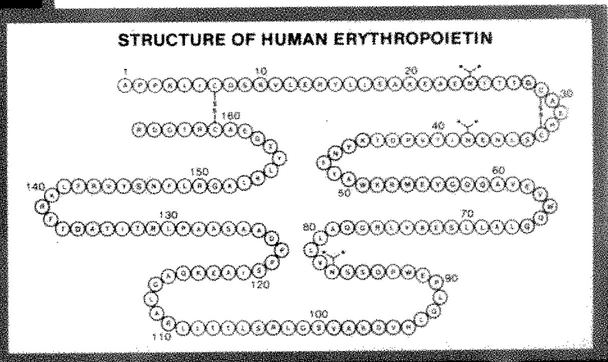

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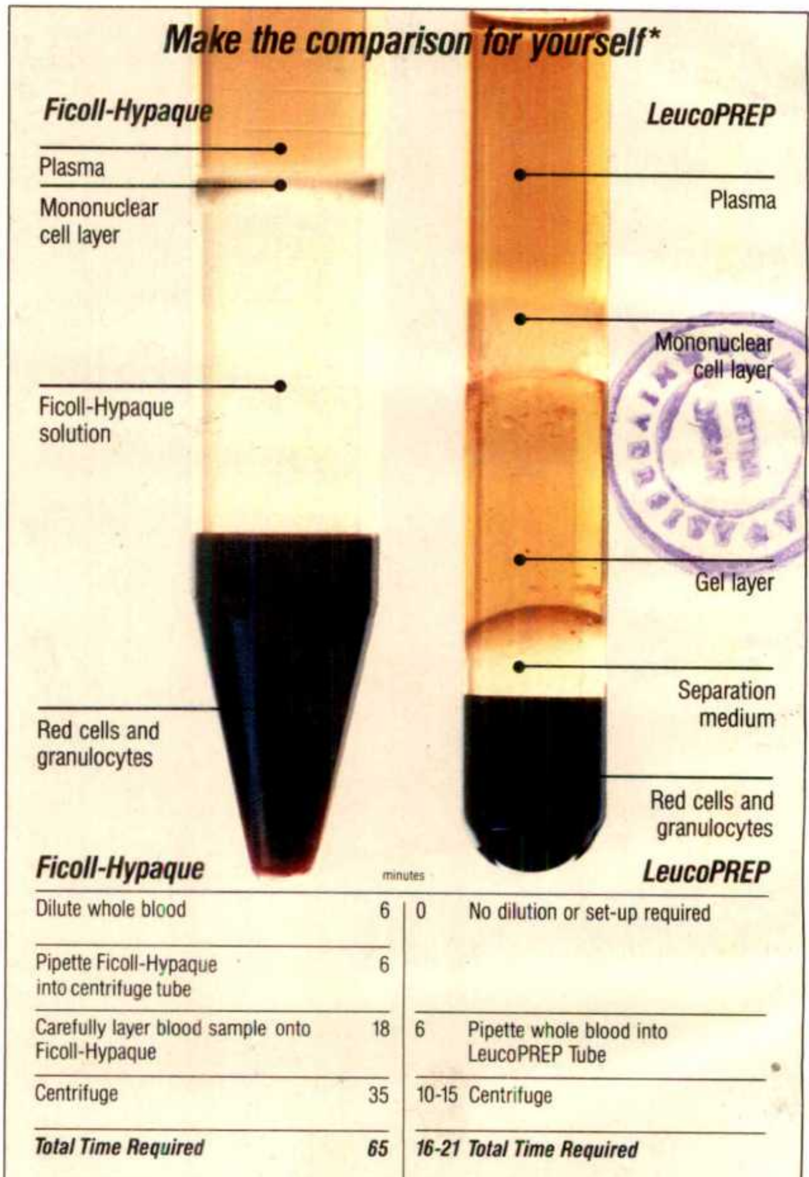

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